

AD _____

Award Number: W81XWH-06-1-0448

TITLE: The Role of Constitutively Active Prolactin Receptors in the Natural History of Breast Cancer

PRINCIPAL INVESTIGATOR: Kuang-tzu Huang, Ph.D.

CONTRACTING ORGANIZATION: University of California at Riverside
Riverside, CA 92521

REPORT DATE: April 2009

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE 1 April 2009		2. REPORT TYPE Annual Summary		3. DATES COVERED 1 Apr 2006 – 31 Mar 2009	
4. TITLE AND SUBTITLE The Role of Constitutively Active Prolactin Receptors in the Natural History of Breast Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-06-1-0448	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Kuang-tzu Huang, Ph.D. E-Mail: khuan001@student.ucr.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California at Riverside Riverside, CA 92521				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Prolactin (PRL) has been implicated as a contributing factor in the incidence of breast cancer. Its cognate receptor (PRLR) is a single-transmembrane receptor that normally requires ligand binding to trigger intracellular signaling. Several isoforms of the human PRLR have been identified, including a long form (LF) and two short forms (SF1a and SF1b). These isoforms share identical amino acid sequences in their extracellular domains, which consist of two structurally-similar subdomains, S1 and S2, but differ in their cytoplasmic domains. Recently, we identified the existence of naturally-occurring S2-deleted (deltaS2) variants of each of these receptors in several human cell lines. These isoforms were constitutively dimerized and activated in the absence of PRL. Because of its potential anti-cancer effects, we focused most of the study on the deltaS2 SF1b isoform. Increased expression of this isoforms in stable cell lines inhibited cell proliferation and migration. When expressed in human breast cancer cells (T-47D) driven by a Tet-responsive promoter, deltaS2 SF1b inhibited both basal and estrogen-stimulated growth. This latter was in part due to decreased serine phosphorylation of estrogen receptor alpha and Akt. Treatment of T-47D cells with a molecular mimic of phosphorylated PRL (S179D PRL) also blocked estrogen-stimulated growth, at least in part through increased expression of both SF1b and ΔS2 SF1b. In primary breast invasive ductal carcinomas, we found loss of SF1b expression in 3 out of 4 tumor samples compared to adjacent normal tissue. The data suggest a physiologically relevant and potentially beneficial role of liganded SF1b and unliganded ΔS2 SF1b receptor. A similar beneficial effect can be achieved by S179D PRL treatment.					
15. SUBJECT TERMS constitutively active prolactin receptor; cell proliferation; cell migration; estrogen; signaling					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	35	19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	8
Conclusion.....	8
References.....	8
Appendices.....	10

INTRODUCTION

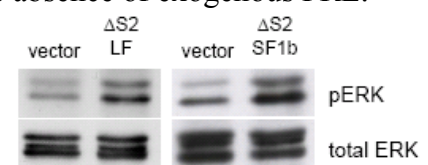
Prolactin (PRL) has been shown to be important in various aspects of the mammary gland development, including proliferation and differentiation (1-3). Due to its proliferative role in normal breast tissue, PRL has also been implicated as a contributing factor in the incidence and progression of breast cancer (4, 5). The actions of PRL are initiated upon its binding to preformed cognate receptors, PRL receptors (PRLRs). Engagement of the ligand leads to conformational changes, triggering intracellular signals through Jak2-Stat5, ERK, PI3K and a number of other pathways (6-9). Several PRLR isoforms have been identified, including the most studied long form (LF), and two short isoforms SF1a and SF1b (10, 11). These isoforms are produced by alternative splicing, resulting in receptors with different lengths in their cytoplasmic domains. The different lengths have been shown to lead to different signaling outcomes. The N-terminal extracellular domain of the receptor, which is identical in primary sequence in all three isoforms, is composed of two structurally-similar, fibronectin-like subdomains, S1 and S2. We have previously shown that deletion of the S2 subdomain results in constitutive dimerization in the absence of PRL (12). We have also demonstrated that S2-deleted (Δ S2), constitutively active isoforms are naturally occurring receptors (12). The objective of this study was to use the Δ S2 PRLRs to determine the individual roles of the different PRLRs and the potential role of the naturally-occurring versions in the natural history of breast cancer. In transiently transfected human breast cancer cells (T-47D), Δ S2 LF has a growth-promoting effect, and both Δ S2 LF and Δ S2 SF1a increase beta-casein expression, in the absence of added PRL. In cancer cells stably expressing Δ S2 PRLRs, we found that one particular isoform, Δ S2 SF1b, inhibits growth and migration. Stable T-47D breast cancer cell lines, whose receptor expression was under the control of a tetracycline responsive promoter, were established. Our results showed that induced expression of Δ S2 SF1b, produced prolonged activation of ERK and up-regulated both the cell cycle inhibitor, p21, and the milk protein, beta-casein, in the absence of added PRL. Expression of Δ S2 SF1b also inhibited estradiol-stimulated cell proliferation. The inhibition of proliferation was at least in part due to decreased phosphorylation of the estrogen receptor (ER) α at serine 118 and Akt at serine 473. We also demonstrated that administration of the pseudo-phosphorylated PRL, S179D PRL, mimicked the effects of the induced Δ S2 SF1b expression. Natural expression of the Δ S2 SF1b form was evaluated by examining tumor samples from patients with invasive ductal carcinomas versus histologically normal contiguous parts from the same patients. Our results showed down-regulation of Δ S2 SF1b in 3 out of 4 pairs. The last part of this report includes the identification of another isoform in which the S2 domain is missing. This isoform, termed SS1, is a soluble receptor and is down-regulated in human breast cancer. SS1 was capable of modulating PRL-stimulated signaling in T-47D cells.

BODY

In this project, we attempted to characterize the biological functions of the naturally occurring, S2 deleted PRLR isoforms. Our previous work has shown that the Δ S2 PRLRs dimerize in the absence of added ligand, as determined by bioluminescence resonance energy transfer (BRET) (12). In an effort to evaluate whether these Δ S2 PRLR isoforms were able to produce intracellular signals, expression plasmids were transfected individually into several human cancer cell lines, including a human breast cancer cell line (T-47D), and PC-3, a prostate cancer cell line that was used in one of the PI's other major projects. ERK phosphorylation was examined since this was a common signaling pathway known to be activated through all three major types of receptors. Results indicated that all three Δ S2 isoforms were able to signal in the absence of exogenous PRL. Examples of Δ S2 LF and Δ S2 SF1b are shown here (right).

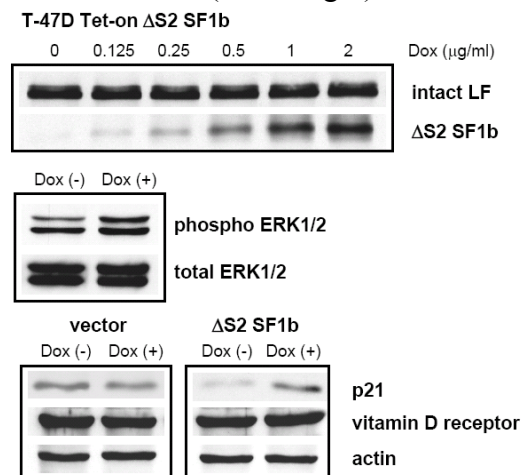
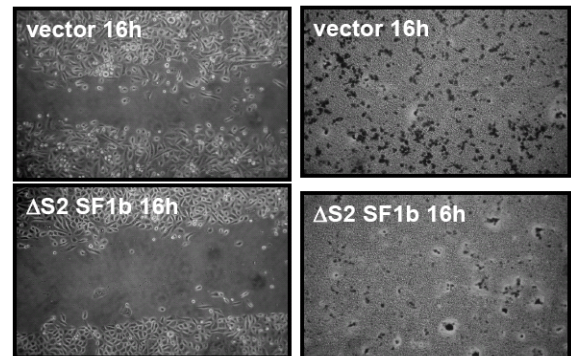
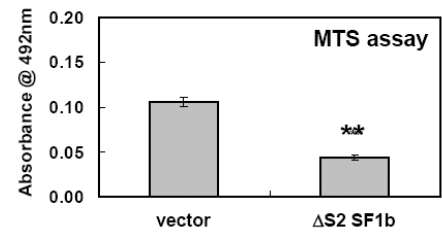
Previous work had suggested that in a transient transfection system, Δ S2 LF had a promoting effect on cell growth, whereas Δ S2 SF1b marginally decreased cell number. This marginal effect may have been due to low transfection efficiency in T-47D cells.

We therefore established stable cell lines. As the figure shows (next page, right), the results showed



a significant decrease in cell growth in cells stably expressing Δ S2 SF1b compared to vector controls.

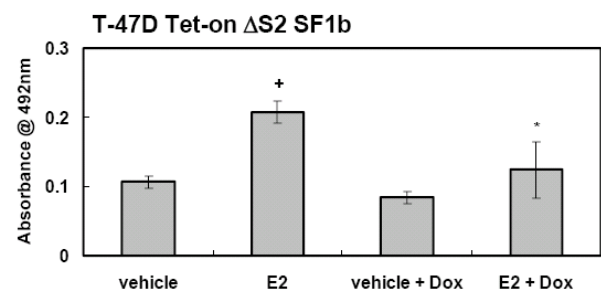
Little is documented about how PRLR isoforms are involved in cell migration although PRL has been shown to be a chemottractant to breast cancer cells (13). We therefore conducted a wound healing (scratch) assay and Transwell migration assay to investigate the change in migratory capacity induced by increased expression of Δ S2 receptors. In the wound healing assay, wounds were introduced with a sterile pipette tip to a monolayer of confluent cells. The extent of wound closure was recorded and quantified after 16 hours. In the Transwell assay, single-cell suspension (in serum-free medium at 10^5 per well) was placed in the upper chamber. The permeable support had a pore size of 8.0 μ m. Medium containing 10% fetal bovine serum was placed in the lower chamber as a chemottractant. After 16 hours, cells were fixed with methanol, and those that had migrated to the lower surface of the membrane were stained and counted. The results showed Δ S2 SF1b-expressing cells exhibit decreased motility compared to vector controls (above right).



Furthermore, in order to further investigate the biological significance of the apparently beneficial Δ S2 SF1b form in breast cancer, we established human breast cancer cells (T-47D) stably expressing the Δ S2 SF1b construct. This construct was controlled by a Tet-responsive transcriptional activator, so that the expression of Δ S2 SF1b could be turned on by tetracycline whenever needed. As shown in the figure (left), cells treated with doxycycline showed a marked, dose-dependent induction of Δ S2 SF1b. A concentration of 1 μ g/ml was sufficient to induce a significant amount of Δ S2 SF1b, apparently comparable to the endogenous level of the intact LF. The dose was therefore chosen for further experiments. After

treatment of the cells with doxycycline and subsequent incubation in low serum medium, immunoblotting showed increased activation of ERK in the absence of added PRL (above left). An increased protein level of p21, was also observed (above left). Also increased were the mRNA level and promoter activity of a milk protein, beta-casein (not shown).

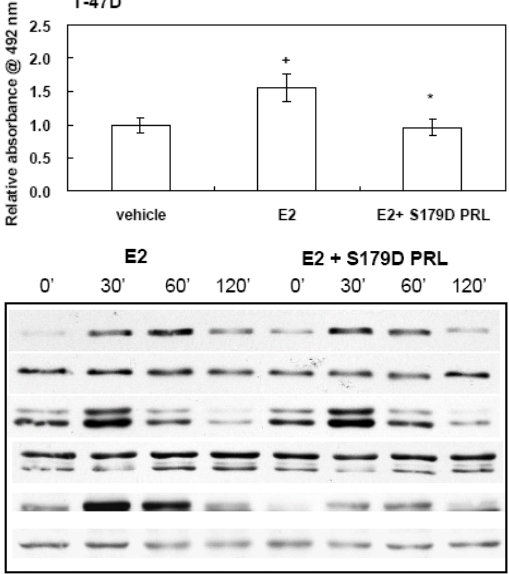
The results shown above indicated a more differentiative outcome with the induction of Δ S2 SF1b. We next examined whether Δ S2 SF1b could also affect estradiol-induced cell proliferation. Cell growth was evaluated by the MTS assay after a three-day incubation with a combination of estradiol and doxycycline. As shown here, a significant increase in viable cell number was observed in cells incubated with estradiol. Induction of Δ S2 SF1b counteracted this effect (right). Control experiments using Tet-On cells stably transfected with an empty vector were also conducted. The cell number remained unchanged in doxycycline-treated cells in response to estradiol (not shown).



We next examined whether the decreased cell number by Δ S2 SF1b induction in E2-treated T-47D cells was due to an alteration in signal transduction. Combinations of E2 and doxycycline or

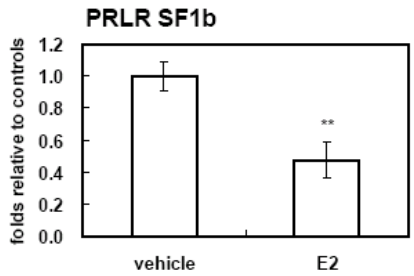
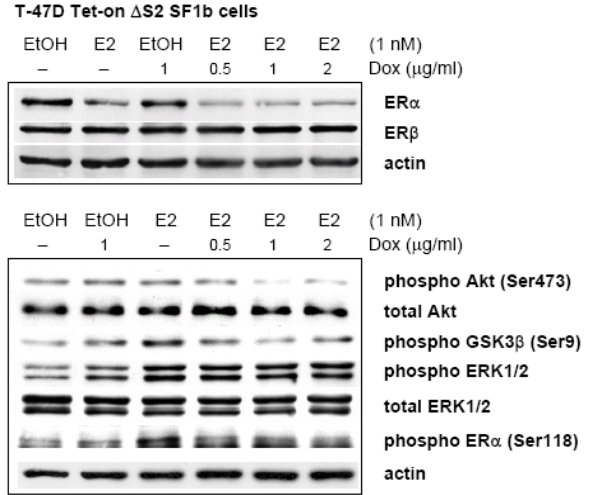
their respective vehicles were administered to the T-47D Tet-On ΔS2 SF1b cells. Western blotting was performed 24 hours after the treatment to observe a “steady-state” result. As shown in the figure (right), E2 treatment decreased the level of ER α, a result previously reported in this and other laboratories (14). Induction of ΔS2 SF1b did not enhance or inhibit this effect. There was no change in ER β levels when cells were treated with either E2 or E2 and doxycycline combined. However, induction of ΔS2 SF1b decreased E2-stimulated phosphorylation of Akt and its downstream kinase, glycogen synthase kinase 3 β (GSK3β), to levels about the same or even lower than the unstimulated state. The induction did not alter the activation of ERK by E2. Parallel control experiments using Tet-On vector cells also showed that doxycycline did not affect the activation of Akt and ERK in response to E2 (not shown).

Previous work from this laboratory has suggested the differential biological effects of unmodified prolactin (PRL) and a molecular mimic of naturally phosphorylated PRL, S179D PRL, on β-casein expression *in vitro*, and mammary gland development *in vivo* (15, 16). We have shown that although apparently bound to the same receptor, S179D PRL creates a different signal towards

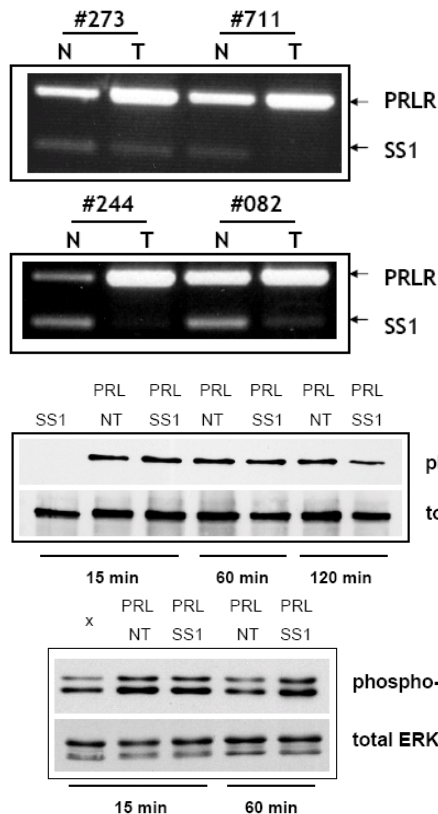


cell differentiation (15, 17, 18). We have reported that PRL enhances the proliferative effect of estradiol (E2) in breast cancer cells (14). We therefore asked what possible effects S179D PRL would have on cell proliferation in response to E2. The experiments were conducted in MCF7 (not shown) and T-47D cells. As shown in the figure (left), T-47D cells were incubated in the presence of E2 or E2 with S179D PRL for 3 days. Viable cell number was also evaluated by the MTS assay. S179D PRL inhibited the growth-promoting effect of E2 in these cells at a concentration as low as 50 ng/ml. Since S179D PRL clearly resulted in inhibition of E2-induced cell proliferation, we next asked whether this molecule had any possible effect on E2-stimulated signaling. As shown (above left), E2 initiated rapid phosphorylation of Akt and ERK but this declined after 30-60 minutes. When the cells were co-incubated in E2 and S179D PRL, the activation of ERK was not affected. However, phosphorylation of ER α at serine 118 was reduced. Serine 118 phosphorylation of ER α positively correlates with its transcriptional activity. Therefore we next examined whether reduced ER α phosphorylation translates to reduced expression of estrogen-responsive genes. Quantitative RT-PCR showed that while incubation of T-47D cells with E2 resulted in increased levels of cyclin D1 and pS2, co-incubation with S179D PRL significantly reduced mRNA levels of these genes (not shown).

We have previously described the pro-differentiative properties of S179D PRL, which occurs partly through up-regulation of the short PRLR isoform, SF1b (17). Our ongoing studies also suggest a role of SF1b in the regulation of cell



proliferation and promotion of differentiation. While S179D PRL increases the expression of SF1b, quantitative RT-PCR analyses revealed that E2 halved SF1b levels in T-47D cells (right on previous page). To examine whether the decrease of SF1b was of any physiological relevance, RT-PCR was performed on cDNA samples from patients with invasive ductal carcinoma (IDC) versus histologically normal contiguous regions. These tissue samples were derived from the Cooperative Human Tissue Network. Decreased expression of SF1b was observed in 3 out of 4 tumor samples (right). SF1b receptor was not detected in one other pair.



In the course of this study, we also identified another isoform in breast cancer cells in which the S2 domain was missing. Sequence analysis of the isolated cDNA revealed an exon 6 deletion in the transcript. This deletion creates a frameshift in the open reading frame, resulting in a shortened soluble receptor essentially composed of half of the extracellular domain. This isoform, designated SS1 (for soluble S1), was first described at the mRNA level by Laud et al (19). They showed that SS1 was highly expressed in one sample of normal mammary tissue and tissue from fibrocystic disease, whereas in mammary tumors, SS1 was expressed at lower levels. In our study, we extended this observation by examining tumor samples from subjects with IDC and samples of adjacent normal tissue from the same patients (see above). Our results from the same four pairs showed higher expression of the intact PRLR in tumor samples and a large complement of SS1 in adjacent normal regions (above left). We were able to isolate the SS1 protein by immunoprecipitation of T-47D conditioned medium with an antibody that recognized the extracellular domain of the receptor (not shown). Given that SS1 is a secreted soluble receptor, we subsequently examined

whether this protein was capable of modulating PRL-induced signaling. Concomitant treatment of T-47D cells with PRL (100 ng/ml) and SS1 conditioned medium (versus control "NT" cells) showed a premature decrease in Stat5 activation at 120 minutes, as determined by immunoblotting (above left). By contrast, SS1 prolonged ERK activation in response to PRL (above left). Thus, SS1 does not act only by sequestering PRL since this would have resulted in reduced activation of both signaling pathways. Rather, it must change the way in which PRL interacts with the receptor. The combination of SS1 and PRL is reminiscent of the way the molecular mimic of phosphorylated PRL signals.

KEY RESEARCH ACCOMPLISHMENTS

- established Δ S2 PRLR and SS1 expression plasmids
- established Δ S2 PRLR stable cell lines (including Tet-On cell lines)
- conducted *in vitro* assays regarding cell proliferation and migration
- delineated signal transduction pathways in estradiol-stimulated breast cancer cells either expressing Δ S2 SF1b or in the presence of pseudo-phosphorylated PRL
- evaluated the expression of receptor SF1b in human breast tumor tissue
- characterized a soluble PRLR (SS1) in breast cancer cell lines and in human primary tissue
- determined the role of SS1 in PRL-stimulated signal transduction in breast cancer cells

REPORTABLE OUTCOMES

Data obtained in the training period (04/2006-03/2009) were presented in poster sessions at the American Society for Cell Biology 2006, the Prolactin and Growth Hormone Gordon Research Conference 2008, and the Department of Defense Breast Cancer Research Program Era of Hope 2008, and are accepted for ENDO 2009. Two papers have been published, one is under revision, and two are being prepared for submission.

1. Tan D, Huang KT, Ueda E and Walker AM. (2008) S2 deletion variants of human PRL receptors demonstrate that extracellular domain conformation can alter conformation of the intracellular signaling domain. *Biochemistry*, 47(1), 479-89.
2. Chen YH, Huang KT, Chen KE and Walker AM. (2009) Prolactin and estradiol utilize distinct mechanisms to increase serine-118 phosphorylation and decrease levels of estrogen receptor alpha in T47D breast cancer cells. *Breast Cancer Res Treat*, in press.
3. Huang KT and Walker AM. Long-term increased expression of the short form 1b prolactin receptor in PC-3 prostate cancer cells decreases cell growth and migration, and causes multiple changes in gene expression consistent with reduced invasive capacity. *The Prostate*, under revision.
4. Ueda E, Huang KT, Nguyen V and Walker AM. Distribution of prolactin receptors supports an important role for local prolactin in mammary gland function. In preparation.
5. Huang KT, Chen YH, Tan D and Walker AM. S179D prolactin blocks estradiol-induced breast cancer cell proliferation in a manner duplicated by signaling from the short (SF1b) prolactin receptor. In preparation.

CONCLUSIONS

PRL has recently been proposed as an important factor in the initiation and progression of various cancers, especially breast cancer. The fact that PRL is an autocrine growth factor not only emphasizes its potential for a contributing role in breast cancer, but also opens new windows for possible new treatment. For example, treatment can be through blockade of autocrine PRL action. Our previous studies have demonstrated that the pseudo-phosphorylated PRL, S179D PRL, inhibits cancer cell proliferation both *in vitro* and *in vivo*, by blocking the autocrine growth loop (20). Another mechanism we have shown is through up-regulation of the pro-differentiative PRLR isoform, SF1b (17). In this project, we discovered a group of naturally occurring PRLR isoforms that, with a truncated extracellular domain, were constitutively active. One of the isoforms, termed ΔS2 SF1b, when overexpressed, inhibited cell proliferation and migration. We also constructed inducible T-47D cell lines. When induced by doxycycline, ΔS2 SF1b inhibited cell proliferation stimulated by estradiol. This was in part due to decreased phosphorylation of ERα at serine 118 and Akt at serine 473. Treatment of T-47D cells with S179D PRL also recapitulated this inhibitory effect through very similar mechanisms. This is very exciting since it demonstrates that S179D PRL blocks not only the effects of autocrine and endocrine PRL, but also the effects of estradiol. When examined in primary breast tumors, we found loss of SF1b expression in most of the tumor samples, suggesting a physiologically relevant and potentially beneficial role of this receptor. Future directions will focus on relationships between S179D PRL treatment and change in levels of receptor isoforms *in vitro* and *in vivo*, and their involvement in estrogen signaling.

REFERENCES

1. Horseman, N. D. Prolactin and mammary gland development. *J Mammary Gland Biol Neoplasia*, 4: 79-88, 1999.
2. Kelly, P. A., Bachelot, A., Kedzia, C., Hennighausen, L., Ormandy, C. J., Kopchick, J. J., and Binart, N. The role of prolactin and growth hormone in mammary gland development. *Mol Cell Endocrinol*, 197: 127-131, 2002.
3. Oakes, S. R., Rogers, R. L., Naylor, M. J., and Ormandy, C. J. Prolactin regulation of

- mammary gland development. *J Mammary Gland Biol Neoplasia*, 13: 13-28, 2008.
4. Vonderhaar, B. K. Prolactin involvement in breast cancer. *Endocr Relat Cancer*, 6: 389-404, 1999.
5. Clevenger, C. V., Furth, P. A., Hankinson, S. E., and Schuler, L. A. The role of prolactin in mammary carcinoma. *Endocr Rev*, 24: 1-27, 2003.
6. Lebrun, J. J., Ali, S., Sofer, L., Ullrich, A., and Kelly, P. A. Prolactin-induced proliferation of Nb2 cells involves tyrosine phosphorylation of the prolactin receptor and its associated tyrosine kinase JAK2. *J Biol Chem*, 269: 14021-14026, 1994.
7. Das, R. and Vonderhaar, B. K. Activation of raf-1, MEK, and MAP kinase in prolactin responsive mammary cells. *Breast Cancer Res Treat*, 40: 141-149, 1996.
8. Bole-Feysot, C., Goffin, V., Edery, M., Binart, N., and Kelly, P. A. Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocr Rev*, 19: 225-268, 1998.
9. Acosta, J. J., Munoz, R. M., Gonzalez, L., Subtil-Rodriguez, A., Dominguez-Caceres, M. A., Garcia-Martinez, J. M., Calcabrini, A., Lazaro-Trueba, I., and Martin-Perez, J. Src mediates prolactin-dependent proliferation of T47D and MCF7 cells via the activation of focal adhesion kinase/Erk1/2 and phosphatidylinositol 3-kinase pathways. *Mol Endocrinol*, 17: 2268-2282, 2003.
10. Hu, Z. Z., Meng, J., and Dufau, M. L. Isolation and characterization of two novel forms of the human prolactin receptor generated by alternative splicing of a newly identified exon 11. *J Biol Chem*, 276: 41086-41094, 2001.
11. Trott, J. F., Hovey, R. C., Koduri, S., and Vonderhaar, B. K. Alternative splicing to exon 11 of human prolactin receptor gene results in multiple isoforms including a secreted prolactin-binding protein. *J Mol Endocrinol*, 30: 31-47, 2003.
12. Tan, D., Huang, K. T., Ueda, E., and Walker, A. M. S2 deletion variants of human PRL receptors demonstrate that extracellular domain conformation can alter conformation of the intracellular signaling domain. *Biochemistry*, 47: 479-489, 2008.
13. Maus, M. V., Reilly, S. C., and Clevenger, C. V. Prolactin as a chemoattractant for human breast carcinoma. *Endocrinology*, 140: 5447-5450, 1999.
14. Chen, Y., Huang, K., Chen, K. E., and Walker, A. M. Prolactin and estradiol utilize distinct mechanisms to increase serine-118 phosphorylation and decrease levels of estrogen receptor alpha in T47D breast cancer cells. *Breast Cancer Res Treat*, 2009.
15. Wu, W., Coss, D., Lorensen, M. Y., Kuo, C. B., Xu, X., and Walker, A. M. Different biological effects of unmodified prolactin and a molecular mimic of phosphorylated prolactin involve different signaling pathways. *Biochemistry*, 42: 7561-7570, 2003.
16. Kuo, C. B., Wu, W., Xu, X., Yang, L., Chen, C., Coss, D., Birdsall, B., Nasser, D., and Walker, A. M. Pseudophosphorylated prolactin (S179D PRL) inhibits growth and promotes beta-casein gene expression in the rat mammary gland. *Cell Tissue Res*, 309: 429-437, 2002.
17. Wu, W., Ginsburg, E., Vonderhaar, B. K., and Walker, A. M. S179D prolactin increases vitamin D receptor and p21 through up-regulation of short 1b prolactin receptor in human prostate cancer cells. *Cancer Res*, 65: 7509-7515, 2005.
18. Wu, W., Chen, Y. H., Ueda, E., Tan, D., Bartolini, P., and Walker, A. M. Different forms of prolactin have opposing effects on the expression of cell cycle regulatory proteins in differentiated mammary epithelial cells. *Oncol Res*, 16: 75-84, 2006.
19. Laud, K., Gourdou, I., Belair, L., Peyrat, J. P., and Djiane, J. Characterization and modulation of a prolactin receptor mRNA isoform in normal and tumoral human breast tissues. *Int J Cancer*, 85: 771-776, 2000.
20. Xu, X., Kreye, E., Kuo, C. B., and Walker, A. M. A molecular mimic of phosphorylated prolactin markedly reduced tumor incidence and size when DU145 human prostate cancer cells were grown in nude mice. *Cancer Res*, 61: 6098-6104, 2001.

APPENDICES

- 1) Research article titled
S2 deletion variants of human PRL receptors demonstrate that extracellular domain conformation can alter conformation of the intracellular signaling domain.
Tan D, Huang KT, Ueda E and Walker AM. (2008) *Biochemistry*, 47(1), 479-89.
- 2) Research article titled
Prolactin and estradiol utilize distinct mechanisms to increase serine-118 phosphorylation and decrease levels of estrogen receptor alpha in T47D breast cancer cells.
Chen YH, Huang KT, Chen KE and Walker AM. (2009) *Breast Cancer Res Treat*, in press.
- 3) Abstract from the research article titled
Long-term increased expression of the short form 1b prolactin receptor in PC-3 prostate cancer cells decreases cell growth and migration, and causes multiple changes in gene expression consistent with reduced invasive capacity.
Huang KT and Walker AM. *The Prostate*, under revision.
- 4) Meeting abstract titled
A naturally occurring, inducible, constitutively active isoform of the human prolactin receptor, ΔS2 SF1b, reduces proliferation and migration in human prostate cancer cells.
Huang KT and Walker AM. (2006) *The American Society for Cell Biology 46th Annual Meeting*.
- 5) Meeting abstract titled
Evidence suggesting that prolactin functions in the mammary duct.
Ueda E, Huang KT, Nguyen V and Walker AM. (2008) *The 2008 Gordon Research Conference: Prolactin and Growth Hormone Family*.
- 6) Meeting abstract titled
Different prolactin receptors mediate different functions in breast cancer cells suggesting the importance of a short and a soluble form to normal breast health.
Huang KT, Tan D and Walker AM. (2008) *Department of Defense Breast Cancer Research Program – Era of Hope 2008*.
- 7) Meeting abstract titled
S179D prolactin blocks estradiol-induced breast cancer cell proliferation, a function duplicated by signaling from the short (SF1b) prolactin receptor.
Huang KT, Chen YH, Tan D and Walker AM. (2009) *The Endocrine Society 91st Annual Meeting*.

S2 Deletion Variants of Human PRL Receptors Demonstrate That Extracellular Domain Conformation Can Alter Conformation of the Intracellular Signaling Domain[†]

Dunyong Tan, Kuang Tzu Huang, Eric Ueda, and Ameae M. Walker*

Division of Biomedical Sciences, University of California, Riverside, California 92521

Received July 14, 2007; Revised Manuscript Received October 14, 2007

ABSTRACT: Using spacers between the C-termini of the long (LF) or short (SF) human prolactin receptors and luciferase/GFP such that bioluminescence resonance energy transfer (BRET) occurred minimally in intact versions of these receptors in the absence of ligand, we have monitored the BRET signal after deletion of regions of the extracellular domain (ECD). Deletion of S2 produced ligand-independent BRET for only those pairings normally occurring in the presence of ligand with the intact receptor. Deletion of the similarly sized S1, or S1 plus S2, produced no ligand-independent or -dependent BRET. When deleted receptors were transfected into human breast (T47D) or prostate (DU145) cancer cells incubated in the absence of added prolactin (PRL) and presence of anti-PRL, expression of the Δ S2LF resulted in increased cell number, whereas expression of the intact receptor did not. When endogenous β -casein expression was examined in T47D cells, the Δ S2LF and Δ S2F1a both showed ligand-independent activation of transcription, again not duplicated by the intact receptor. Paired with evidence in the literature for predimerization of PRLRs, these results demonstrate that altered ECD conformation, and not just a change in bulk, produces altered conformation of the intracellular signaling region of the receptors, supporting the concept that ligand binding to the ECD of intact predimerized receptors could initiate signaling. In addition, the current work supports a dual proliferative and differentiative role for the LF receptor, but only a differentiative role for the SF1a receptor. Naturally occurring Δ S2 PRL receptors (PRLR) were also found in normal and cancerous human cells. This additionally suggests a heretofore unappreciated ligand-independent role for PRLRs.

Prolactin (PRL)¹ is a pituitary polypeptide hormone characterized by multiple biological actions including growth control in the prostate and stimulation of development and milk protein gene expression in the mammary gland (1–3). The effects of PRL are mediated by interaction with specific receptors located on the plasma membrane of many target tissues (4, 5). PRL receptors (PRLR) belong to the superfamily of hematopoietic cytokine receptors, which are devoid of intrinsic catalytic activity (6). Rather, family members associate with tyrosine kinases that are activated upon binding of the ligand, which in the case of the PRLR, results

in one ligand–two receptor ternary complexes (7). Activation requires close proximity of the intracellular domains (7) and can be measured by bioluminescence resonance energy transfer (BRET), as described previously (8, 9). Receptors in this family have an extracellular ligand-binding domain, a single hydrophobic transmembrane domain, and an intracellular domain (10). The extracellular domain (ECD) of the PRLR (and other members of this cytokine receptor family) consists of two subdomains of approximately 100 amino acids each, S1 and S2, both of which are involved in ligand binding (10, 11). Increasing evidence shows that the two subdomains of the ECD function in very different ways. Gourdou and co-workers (12) examined the properties of mutant forms of the rabbit long form PRLR, in which the S1 or S2 subdomains were deleted, and found deletion of S2 made the receptor constitutively active when using milk protein gene expression as the measure of bioactivity. Lee et al. (13) also showed that deletion of most of the ECD (but not specifically the S2 region) of the human long PRLR led to constitutive activation and ligand-independent proliferation of cells. These investigators produced such mutant forms to analyze regions of the ECD involved in ligand binding and the process of receptor dimerization.

More recent work on the PRLR, however, has shown that the receptors are predimerized (14–16), a conclusion previously drawn for several other members of this receptor

[†] This work was supported by California Breast Cancer Research Program Grant 10PB-0127, NIH Grant DK61005, and by a grant from the Prostate Cancer Foundation. K.T.H. was a recipient of an individual predoctoral fellowship from the Department of Defense Breast Cancer Research Program - BC051103.

* Corresponding author. Telephone: 951-827-5942. Fax: 951-827-5504. E-mail: ameae.walker@ucr.edu.

¹ Abbreviations: PRL, prolactin; PRLR, prolactin receptor; LF, long form; SF, short form; GFP, green fluorescent protein; BRET, bioluminescence resonance energy transfer; ECD, extracellular domain; Rluc, *Renilla* luciferase; Gluc, *Gaussia* luciferase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; PMS, phenazine methosulfate; Δ S1, receptor missing the S1 region of the extracellular domain; Δ S2, receptor missing the S2 region of the extracellular domain; Δ S1S2, receptor missing both the S1 and S2 regions of the extracellular domain; DPBS, Dulbecco's phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium.

superfamily (17, 18). It is thus suggested that the binding of ligand initiates signal transduction by causing a change in the conformation of the preformed receptor pair. In the current study, we have used the generation of a BRET signal to examine whether a change in conformation of the ECD could in fact result in a change in conformation of the intracellular, signaling region. Binding of PRL to the PRLRs involves parts of both the S1 and S2 regions of the ECD (7, 10, 11, 19). We therefore deleted either the S1 or similarly sized S2 region of the ECD (or both). In addition, we used the LF and both SFs (SF1a and SF1b) of the human PRLR to take advantage of our previous determination that while all homo- and most heteropairs of these receptors form a signaling complex with PRL (8), SF1a and SF1b heteropairs do not.

The current study demonstrates that removal of the S2 extracellular subdomain changes the conformation of the intracellular signaling region of the LF and both SF receptors, thereby supporting the concept that the conformation of the ECD can affect the conformation of the intracellular domain of these single transmembrane receptors, and hence that ligand binding could initiate signaling by this means in predimerized receptors. In addition, the current work supports a dual proliferative and differentiative role for the LF receptor, but only a differentiative role for the SF1a receptor. Moreover, the finding that the Δ S2 versions are produced naturally in human prostate and endothelial cells suggests a heretofore unappreciated ligand-independent role for Δ S2 PRLRs in these tissues.

EXPERIMENTAL PROCEDURES

Materials. The codon-humanized pGFP²-N1 (h) and pRluc-N1 (h) vectors, containing multiple cloning sites upstream of the GFP² gene or Rluc gene, and the luciferase substrate, DeepBlueC, were purchased from Perkin-Elmer (Wellesley, MA). Coelenterazine *h*, another substrate for Rluc used for the spectral studies and receptor binding assays, was purchased from Assay Designs, Inc. (Ann Arbor, MI). The PRLR cDNAs, encoding the LF, SF1a and SF1b, were originally kindly provided by Dr B. K. Vonderhaar (NCI, Bethesda, MD) and were cloned into the GFP and Rluc vectors as described previously (8). The cell proliferation assay reagents, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) and phenazine methosulfate (PMS), were products of Promega (Madison, WI). Reverse transcriptase and pfu polymerase were purchased from Stratagene (La Jolla, CA). The transfection reagent, lipofectamine 2000, was from Invitrogen (Carlsbad, CA). Unmodified PRL was purified from *Escherichia coli* (BL21) as described previously (20).

Generation of Eukaryotic Expression Vectors for PRLR Mutants Tagged with GFP² or Rluc. The cDNA for the human PRLR in a mammalian expression vector has been described previously (8). Mutants were established by PCR splicing. As shown in Figure 1, to create Δ S1 or Δ S2 forms of the PRLRs, 101 amino acids (303 bp nucleotide) were deleted (amino acid 3–103 in S1 or 106–206 in S2). Furthermore, to create Δ S1S2, 204 amino acids (3–206) were deleted. Four primers (Table 1) were required for each construct, two flanking primers (primers 2 and 3) and two hybrid primers (primers 1 and 4). The two flanking primers

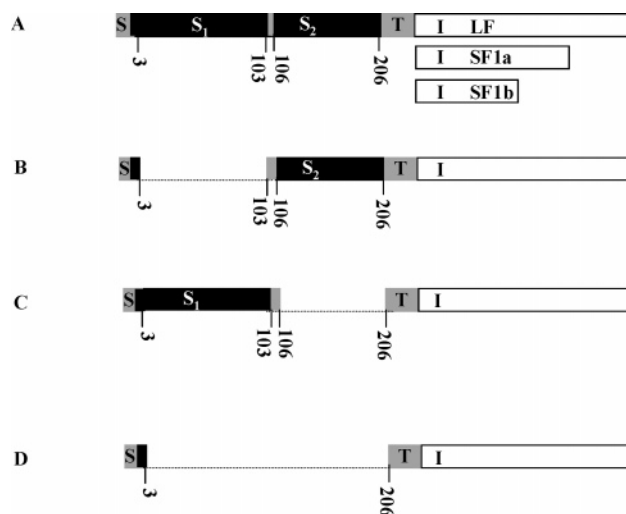


FIGURE 1: Diagram illustrating the different versions of the PRLR used. (A) Intact human LF PRLR with the shorter intracellular regions of SF1a and SF1b illustrated. The transmembrane and ECD regions are the same for all three. (B) S1- deleted. (C) S2-deleted. (D) S1- and S2-deleted. The dotted line illustrates the part deleted, and the numbers given are the amino acids in the mature sequence. S, signal peptide; S1, subdomain 1; S2, subdomain 2, of the extracellular domain; T, transmembrane; I, intracellular part of LF, SF1a and SF1b.

were based on the sequence immediately before and after the part to be deleted, and primers 2 and 3 were complementary to each other. In this study, 15 bases were designed on each side of the sequence to be deleted and combined with a 30 base primer in the next PCR reaction to generate fragments with deletion of the S1, S2 sequence, or both.

Additionally, to generate these deleted PRLRs tagged with Rluc or GFP² at the carboxy terminus, forming Δ S1 PRLR-GFP², Δ S2 PRLR-GFP², Δ S1S2 PRLR-GFP², and Δ S1 PRLR-Rluc, Δ S2 PRLR-Rluc, Δ S1S2 PRLR-Rluc, the extra DNA base pairs corresponding to Mlu I (ACGCGT) and Kpn I (GGTACC) were designed to be upstream of the initiation codon on the forward primer 1 and to replace the stop codon on the reverse primer 4 (the nucleotide in bold in Table 1). An extra triplet ACC was also designed to be immediately prior to the initiator codon, ATG, to facilitate and therefore increase the translation rate in transfected mammalian cells. The high fidelity proofreading DNA polymerase (pfu polymerase) from Stratagene was used. The original plasmids, pEF6-LF, pEF4-SF1a, and pCR2.1-SF1b, which contain the entire coding sequence for LF (1866 bp), SF1a (1128 bp), and SF1b (864 bp), respectively, were used as template in the first stage PCR.

Cell Culture and Transfection. Human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) containing high glucose, 1 mM sodium pyruvate and pyridoxine hydrochloride, 2 mM L-glutamine, supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 0.7 μ M streptomycin. The cells were seeded at a density of 5×10^5 cells per well of a six-well (or as indicated) plate. Transient transfections were performed the following day when the cells were 90–95% confluent using Lipofectamine 2000 (Invitrogen) in accordance with the protocol provided by the vendor. Briefly, 3.5–4.5 μ g of DNA was used per well (depending on the experiment). The DNA was initially incubated in 250 μ L of Opti-MEMI (Invitrogen) (without

Table 1: Flanking (2,3) and Hybrid (1,4) Primers Used in PCR Splicing^a

construct	flanking primers	hybrid primers
ΔS1-LF	2 5'-ctccaaaggagggtctactgtccattcag-3' 3 5'-ctgaatggacagtttagacctcttggag-3'	1 5'-gac acgcgt accatgaaggaaatgtg-3' 4 5'-aac gggtacc agtgaaaggagtgtg-3'
ΔS1-SF1a	2 5'-ctccaaaggagggtctactgtccattcag-3' 3 5'-ctgaatggacagtttagacctcttggag-3'	1 5'-gac acgcgt accatgaaggaaatgtg-3' 4 5'-aac gggtacc agtgactgtgtgtaa-3'
ΔS1-SF1b	2 5'-ctccaaaggagggtctactgtccattcag-3' 3 5'-ctgaatggacagtttagacctcttggag-3'	1 5'-gac acgcgt accatgaaggaaatgtg-3' 4 5'-aac gggtacc aggggtcacctcaa-3'
ΔS2-LF	2 5'-tgtatcattcatggttagggtctgctgaac-3' 3 5'-gttcagccagaccctaccatgaatgataca-3'	1 5'-gac acgcgt accatgaaggaaatgtg-3' 4 5'-aac gggtacc agtgaaaggagtgtg-3'
ΔS2-SF1a	2 5'-tgtatcattcatggttagggtctgctgaac-3' 3 5'-gttcagccagaccctaccatgaatgataca-3'	1 5'-gac acgcgt accatgaaggaaatgtg-3' 4 5'-aac gggtacc agtgactgtgtgtaa-3'
ΔS2-SF1b	2 5'-tgtatcattcatggttagggtctgctgaac-3' 3 5'-gttcagccagaccctaccatgaatgataca-3'	1 5'-gac acgcgt accatgaaggaaatgtg-3' 4 5'-aac gggtacc aggggtcacctcaa-3'
ΔS1S2-LF	2 5'-tgtatcattcatggttagggtctgctgaac-3' 3 5'-ctgaatggacagtttagacctcttggag-3'	1 5'-gac acgcgt accatgaaggaaatgtg-3' 4 5'-aac gggtacc agtgaaaggagtgtg-3'
ΔS1S2-SF1a	2 5'-tgtatcattcatggttagggtctgctgaac-3' 3 5'-ctgaatggacagtttagacctcttggag-3'	1 5'-gac acgcgt accatgaaggaaatgtg-3' 4 5'-aac gggtacc agtgactgtgtgtaa-3'
ΔS1S2-SF1b	2 5'-tgtatcattcatggttagggtctgctgaac-3' 3 5'-ctgaatggacagtttagacctcttggag-3'	1 5'-gac acgcgt accatgaaggaaatgtg-3' 4 5'-aac gggtacc aggggtcacctcaa-3'

^a Note: The nucleotides in bold are restriction sites designed to ligate into eukaryotic expression vector GFP²N1 or Rluc N1.

serum and antibiotics) and mixed gently. A 1:25 dilution of Lipofectamine 2000 in 250 μ L of the same medium was then prepared. After a 5-min incubation at room temperature, the diluted DNA and Lipofectamine 2000 solutions were mixed and incubated at room temperature for 20 min to allow the DNA–Lipofectamine2000 complexes to form. The complexes were then added to the cells (in medium with serum but without antibiotics) and mixed by rocking the plate back and forth. After a 48-h incubation of the cells at 37 °C in a CO₂ incubator, the cells were subjected to BRET analysis as described previously (8).

Confocal Imaging. Confocal microscopy (using a Zeiss 510) was applied to check cellular expression and localization of GFP²-tagged PRLRs. HEK293 cells were plated at a density of 5×10^5 cells/35-mm well on polylysine-coated coverslips ($\Phi = 12$ mm) and cultured in DMEM as described earlier. One day following plating, when the cells reached about 90% confluency, the cells were transfected with 0.8 μ g of DNA/35-mm well using lipofectamine 2000 as described earlier. Forty-eight hours after transfection, microscopic observation was performed.

Living Cell-Based Binding and Uptake Study. To investigate the binding/uptake properties of the deleted receptors in living cells, we developed a luminescence-based method. Briefly, the cDNA coding sequences of PRL and *Gaussia* luciferase (Nanolight technologies, Pinetop, AZ), a luciferase of 17 kDa, were amplified and fused from the original plasmid PT7-SCII-prl and pcDNA-3.1Gluc based on a PCR approach. A spacer coding for a 14 amino acid linker in the fusion protein was also included. The sequence of the spacer was GSRYRGPGLPVAT. The whole fusion fragment cDNA (prl-Gluc) was then subcloned into a mammalian expression vector, pcDNA3.1, which additionally codes for the signal sequence. Finally, transfection of pcDNA3.1-prl-(Gluc) into 293 cells was carried out, and conditioned medium (using horse serum so that the conditioned medium could be assayed in the Nb2 bioassay) containing PRL-Gluc was collected. The PRL bioactivity of the fusion protein was determined by Nb2 assay, as previously described (20, 21). A dilution of conditioned medium equivalent to 50 ng/mL of unmodified PRL in the same bioassay was chosen to compare binding and uptake abilities at physiological

concentration. To assess binding/uptake, cells were incubated in conditioned medium (diluted with fresh culture medium containing serum to achieve the correct concentration) for 3 h in the absence and presence of a 50-fold excess of unmodified PRL. Binding/uptake in the presence of excess PRL were considered nonspecific. A separate, nonspecific binding/uptake control was run for each receptor type. The cells were then washed three times with PBS before incubation in BRET² buffer (Dulbecco's PBS containing 0.9 mM CaCl₂, 0.5 mM MgCl₂·6H₂O, and 5.5 mM D-glucose) for 5 min and measurement of luminescence intensity.

RNA Isolation, RTPCR, and Western Blot. Forty-eight hours after transfection with intact or deleted PRLR, the T47D cells were washed three times with Dulbecco's PBS, and RNA extractions were performed using TRIzol reagent (Invitrogen) according to the protocol suggested by the vendor. Total RNA was used for first-strand synthesis of cDNA using reverse transcriptase (Stratagene, La Jolla, CA). Briefly, 5 μ g of total RNA and 3 μ L of random primer (Epicentre, Madison, WI) in nuclease-free water were incubated at 65 °C for 5 min and slowly cooled to room temperature to allow the primers to anneal to RNA. Then, 5 μ L of $5 \times$ RT buffer, 1 μ L of RNase inhibitor, 2 μ L of 100 mM dNTP mix, and 1 μ L of reverse transcriptase (Stratagene) were added and mixed and incubated at 42 °C for 1 h. For PCR, 1 μ L of RT product was added to a 50- μ L reaction containing 5 μ L of $10 \times$ pfu polymerase buffer, 4 μ L of dNTP (4 mM), 2.5 μ L of forward or reverse primer (10 mM), and 1 μ L of pfu polymerase. β -Casein mRNA was detected using the following two primers: 5'-acactgtctacactaaggc-3' (forward) and 5'-tgctgtagtcacagggtaga-3' (reverse), which yield a 303 bp fragment of the β -casein open reading frame. PCR parameters were as follows: 95 °C for 1 min, 55 °C for 50 s, 72 °C for 2 min, with a final extension period of 72 °C for 10 min. β -Casein mRNA is normally expressed at very low levels in these cells. Cycle titration ensured amplification in the linear range and a semiquantitative result. All data were normalized to β -actin.

When detecting the presence of the ΔS2 receptor in prostate cancer and endothelial cells, PCR primers with the following sequences were utilized: 5'-atcatgatggctaatgccacta-3' (forward) and 5'-tccaacagatgagcatcaaatc-3' (reverse).

Amplifications were carried out for 35 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 45 s. A final extension at 72 °C was performed after the last cycle for 5 min. For detection of the different isoforms of Δ S2 PRLR, the same forward primer was used, but isoform-specific reverse primers were utilized: 5'-tggggttcctcacactttc-3' (LF), 5'-gtaatgagaggcaccacacat-3' (SF1a), and 5'-aggggtcacctccacagat-3' (SF1b). Cycle number differed (see Figure 1 caption). The final PCR product was resolved in a 1% agarose gel, stained with ethidium bromide, and visualized under UV light. All bands obtained were sequenced to confirm their identity.

For Western blot, whole cell lysates were collected and 500 μ g of protein were precleared with 1 μ g of normal mouse IgG and 20 μ L of protein A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA). Precleared lysates were then incubated with 1 μ g of mouse anti-PRLR ECD antibody (Zymed, Carlsbad, CA) at 4 °C overnight. Immunocomplexes were captured with protein A/G agarose, and the beads were washed 3 times before dissolution of attached proteins in SDS loading buffer. SDS gel separation, transfer, membrane blocking, and incubation in anti-PRLR were as described previously (22), using an antibody dilution of 1:1000. Immunoprecipitation with normal mouse IgG served as the negative control.

Cell Proliferation Assay (MTS Assay). This assay was performed under stringent conditions previously described (21). Briefly, 42 mg of MTS reagent powder was dissolved in 21 mL of DPBS and filter-sterilized using a 0.2- μ m filter. To this was added 100 μ L of PMS/2 mL MTS solution under light-protected conditions. Twenty microliters of the freshly combined MTS/PMS solution was added to each well of a 96-well assay plate containing 100 μ L of cells in culture medium without serum. The cells plus reagents were then incubated at 37 °C, 5% CO₂ for 3 h, after which time the absorbance at 490 nm was recorded using a microplate spectrophotometer. The number of cells was in the linear range of the assay.

Fluorescence and BRET² Measurements. Transfected cells were harvested within 48 h of transfection by washing with DPBS (three times), detaching in DPBS containing 2 mM ethylenediaminetetraacetic acid (EDTA), pelleting by centrifugation, and resuspension in BRET² buffer (DPBS containing 0.9 mM CaCl₂, 0.5 mM MgCl₂·6H₂O, and 5.5 mM D-glucose) at a density of 2×10^6 /mL. After this and before the BRET² measurements, the cells were incubated at 37 °C for 1 h. For each measurement, 0.5 mL of the cell suspension was loaded into a 0.5-cm² quartz cuvette. Fluorescence and bioluminescence spectral scanning were performed using a FluoroMax-2 spectrofluorometer. Bioluminescence scanning and BRET² signal detection were carried out immediately after the addition of 5 μ M of the cell permeant luciferase substrate, DeepBlueC, with a black card in the path of the excitation light beam. Data were collected with the slits set at 5 nm, datum points were collected at 5-nm intervals, and signal integration was for 0.5 s per datum point. Energy transfer was defined as the BRET ratio $(\text{Emission}_{500\text{nm}-520\text{nm}} - \text{background}_{500\text{nm}-520\text{nm}}) / (\text{Emission}_{385\text{nm}-420\text{nm}} - \text{background}_{385\text{nm}-420\text{nm}})$. The signals obtained from nontransfected cells were considered background.

Statistical Analysis. All experiments were repeated a minimum of three times, and most were repeated five or six times. Numerical data are presented as the mean \pm SEM, and statistical significance was determined by ANOVA with post-tests using Bonferroni corrections for multiple comparisons against a single control, where appropriate.

RESULTS

Construction of Eukaryotic Expression Vectors for ECD-Mutated PRLRs Tagged with GFP² or Renilla Luciferase. A set of ECD mutant receptors was constructed using a rapid splicing PCR approach. For each receptor, we produced three mutant forms: S1 deletion, S2 deletion, or a form with both S1 and S2 deleted, named Δ S1, Δ S2, or Δ S1S2, respectively (Figure 1). Mutant sequences were confirmed by sequencing. To detect close approximation of the receptors, we used BRET and hence each mutant receptor was tagged with either *Renilla* luciferase (Rluc), which acts as an energy donor, or a variant of green fluorescence protein (GFP²), as an energy acceptor. The luciferase or GFP² was separated from the C termini of the receptors by 13 or 12 amino acid linkers, respectively. Insertion of these linkers has been previously shown to prevent most BRET in the intact receptors in the absence of ligand (8 and figure herein). Eighteen plasmids were constructed: Δ S1LF-GFP², Δ S2LF-GFP², Δ S1S2LF-GFP², Δ S1SF1a-GFP², Δ S2SF1a-GFP², Δ S1S2SF1a-GFP², Δ S1SF1b-GFP², Δ S2SF1b-GFP², Δ S1S2SF1b-GFP², Δ S1LF-Rluc, Δ S2LF-Rluc, Δ S1S2LF-Rluc, Δ S1SF1a-Rluc, Δ S2SF1a-Rluc, Δ S1S2SF1a-Rluc, Δ S1SF1b-Rluc, Δ S2SF1b-Rluc, and Δ S1S2SF1b-Rluc. Energy transfer occurs when the Rluc and GFP² molecules come within ~ 100 Å of one another and is initiated by the addition of the Rluc substrate, DeepBlueC, as described previously (8, 9).

To verify expression and comparable localization of the receptors, confocal fluorescence microscopy and spectrofluorimetry were performed on HEK293 cells 48 h post-transfection with the GFP² plasmids. As shown in Figure 2A, a proportion of each expressed transfected receptor translocated to the region of the plasma membrane after synthesis. Because the receptors are synthesized in the rough endoplasmic reticulum and travel through the Golgi on their way to the surface, regions of the cell consistent with these intracellular organelles were also highly fluorescent. Similar subcellular distributions were observed in human breast cancer T47D cells and prostate cancer DU145 cells (data not shown). Intracellular distribution was very similar to GFP² tagged versions of the intact receptors previously analyzed (8).

To verify normal fluorescence and bioluminescence spectral properties of the GFP² and Rluc, when attached to all versions of the receptors, measurements were performed using a spectrofluorometer, as described previously for the intact receptors (8, 9). All GFP²- and Rluc-tagged receptors had normal spectral properties. The spectra for the Δ S1 forms are shown as an example (Figure 2B). On the basis of amount of fluorescence and luciferase activity, one can appreciate that transfection with equal amounts of cDNA results in different degrees of expression of each receptor, with SF1b being most efficiently expressed, followed by the LF and SF1a, the same as previously reported for the intact receptors,

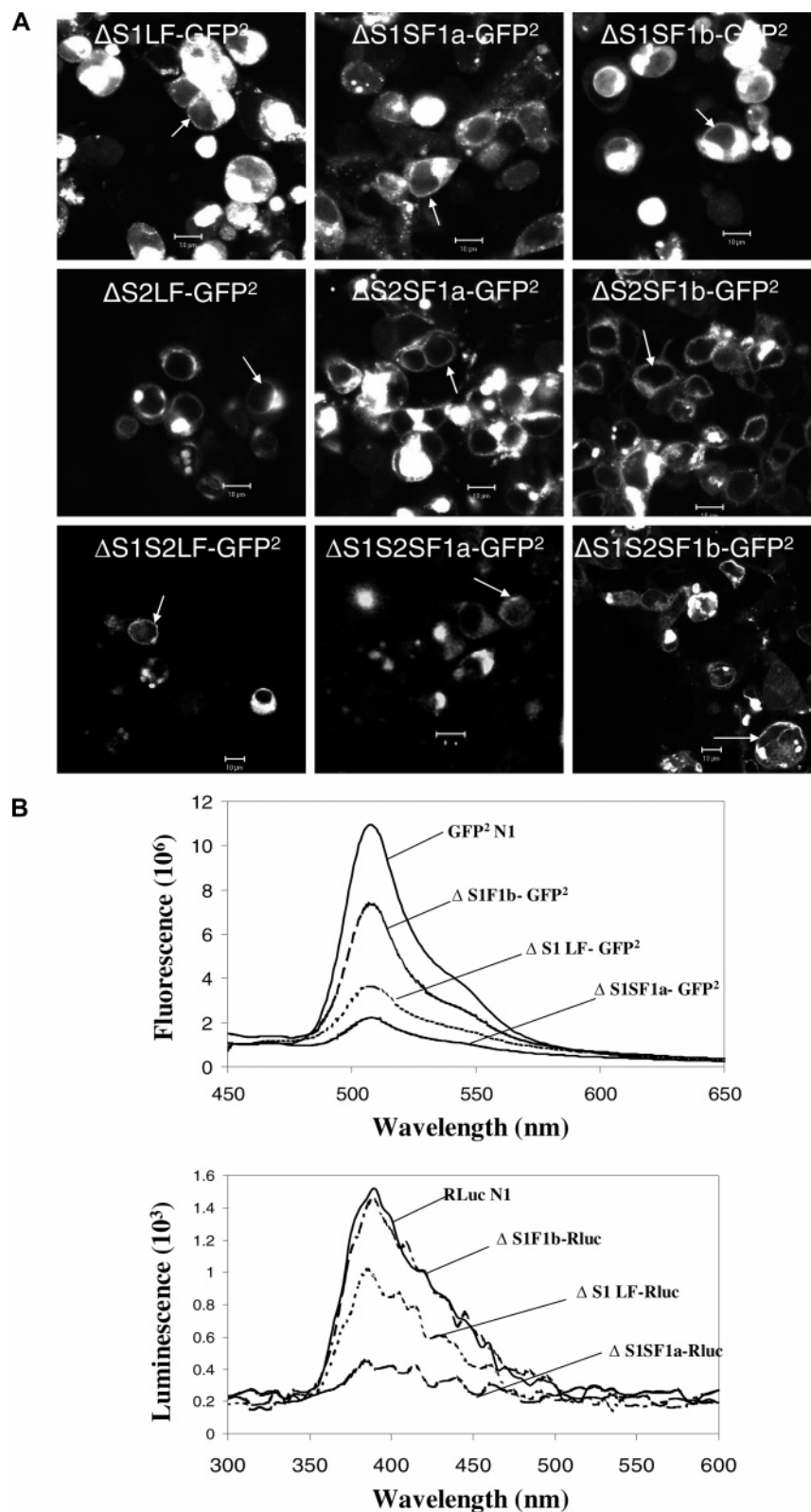


FIGURE 2: Confocal images demonstrating expression, localization (A), and spectral properties (B) of the GFP² and/or RLuc-tagged deleted human PRLRs. HEK293 cells transiently transfected with plasmids containing cDNA coding for the receptors tagged with GFP² were examined by confocal microscopy after 48 h. Bar, 10 μ m; white arrowhead, localization of the tagged receptors to the region of the plasma membrane. For spectral scanning, GFP² was excited at 405 nm. Bioluminescence generated by RLuc was detected in the presence of substrate, DeepBlueC (5 μ M), with blockade of the external excitation light source. Only the spectra for the Δ S1 versions are shown by way of example, but all spectra were unaltered by attachment to the various receptors. GFP²N1 is the GFP² expressing vector without any receptor, and RLucN1 is the luciferase expressing vector without receptor. The relative expression of each receptor can be appreciated by comparing the amount of fluorescence or luciferase activity. Relative expression levels were the same for the intact and deleted versions of the receptor.

either untagged or similarly tagged (8). The different degrees of expression were not a function of transfection efficiency

since transfection efficiency was indistinguishable among constructs on any given occasion (data not shown). Also,

all ECD versions of each receptor tested in the current experiments were expressed at the same level (i.e., intact, Δ S1, Δ S2, and Δ S1S2), and each had the described same relative expression (i.e., SF1b > LF > SF1a). In other words, only the intracellular region of the receptor appeared to influence the level of protein expression.

S1 or S2 Deletion Decreased Binding and Uptake of the Ligand. To investigate the binding and uptake properties of the deleted receptors in living human cells (HEK293) under physiological conditions with homologous ligand, we developed a luminescence-based approach in which a low molecular weight luciferase was attached to the C-terminus of human PRL (see Experimental Procedures). This assay was conducted at 37 °C to ensure normal interaction of the ligand with the ECD and normal movement of the receptors in the lipid bilayer. The PRL-Gluc construct was expressed in human cells (HEK293), and conditioned medium (contained no FBS) was used as the source of ligand for binding. Biological activity of the Gluc-tagged human PRL was assessed by Nb2 PRL bioassay and compared to untagged human PRL, as shown in Figure 3A. The PRL-Gluc showed a concentration-related ability to stimulate Nb2 cell proliferation, which reached the same maximum as untagged PRL.

For binding/uptake studies, 50 ng/mL PRL activity equivalents of PRL-Gluc were incubated with the cells for 3 h at 37 °C, the cells were then washed with PBS three times, and the relative cell-associated luciferase activity was measured. Nonspecific binding/uptake (which was subtracted from the total and separately measured for each receptor construct) was assessed by binding/uptake in the presence of a 50-fold excess of unmodified PRL (2.5 μ g/mL). The results showed that deletion of the S1 or S2 subdomain markedly decreased binding/uptake for each of the receptors (Figure 3B). Although consistently higher, this level of binding/uptake was not statistically different from nonspecific. The 3-h incubation period was used to rigorously test for binding. Although this means that there was also uptake by the cells, the logic was that both binding and uptake are receptor-mediated processes and hence that the long incubation should amplify any specific binding.

Prolactin-Independent BRET in Homo- and Heteropairs of Δ S2 Receptors. To assess approximation of the intracellular regions of the deleted receptor forms, BRET was measured. In this technology, when the energy donor and acceptor protein come within 100 Å of each other, energy transfer from donor to acceptor occurs. The amount of energy transfer is given as the BRET ratio, defined in Experimental Procedures. In the absence of PRL, there was a small background BRET ratio for the intact homopaired receptors (Figure 4A). This was unchanged by S1 deletion. S2 deletion on the other hand increased the BRET ratio to a level more than 3-fold background with the intact receptors. Deletion of both S1 and S2 resulted in no significant change from the intact receptors (Figure 4A). Only the Δ S2 versions were therefore tested for the receptor heteropairs (Figure 4B). For Δ S2LF- Δ S2SF1a, the BRET ratio was 4-fold that of the intact receptor. For Δ S2LF- Δ S2 SF1b, the BRET ratio was double the intact receptor. Addition of PRL to Δ S1, Δ S2, and Δ S1S2 homopairs did not change the BRET ratios of these deleted mutants (Figure 4C), thereby illustrating a lack of significant effect of any potential residual PRL binding in these receptors. Although described sequentially as

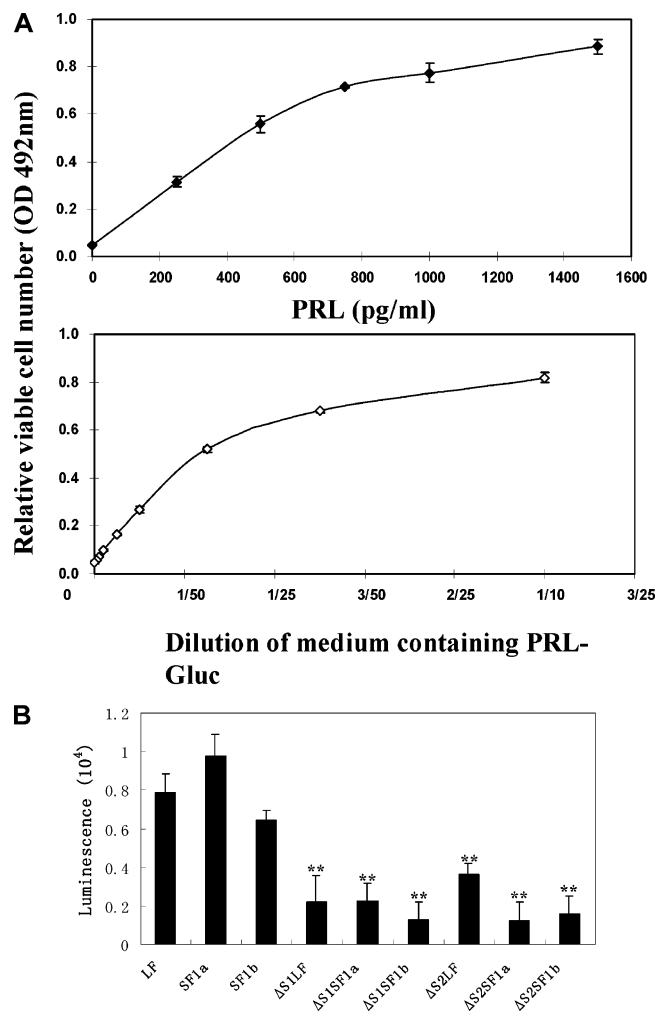


FIGURE 3: Biological activity of PRL-Gluc and the relative ability of the intact and deleted PRLRs on HEK293 cells to bind and internalize PRL-Gluc. Panel A compares diluted conditioned medium with unmodified human PRL. PRL-Gluc was biologically active, reaching the same maximum as unmodified PRL. OD 492 nm is absorbance in the MTS assay and is representative of relative cell number. A dilution of conditioned medium with bioactivity equivalent to 50 ng/mL unmodified PRL was used for the studies in panel B. After a 3-h incubation at 37 °C, the cells were washed three times and cell-associated luminescence was determined. Nonspecific binding/uptake (binding/uptake in the presence of excess unmodified PRL for each receptor type) was subtracted from each result. **, significantly different from their intact counterpart with $p < 0.01$.

knowledge developed, the data presented in Figure 4A–C were obtained on the same occasion.

Effect of the Deleted PRLRs on Breast and Prostate Cancer Cell Growth and Endogenous β -Casein Gene Transcription. To assess the biological result of the deletions, receptors were transiently transfected into human breast (T47D) or prostate (DU145) cancer cells. Once again, transfection efficiency of each ECD form of the receptor was the same, but expression levels of the protein were different with the same relative expression levels as for the HEK293 cells. Forty-eight hours post-transfection, the cells were cultured for a further 24-h period in fresh medium in the continued absence of added PRL. Cell number was then assessed by MTS assay in serum-free medium (21), or RNA was extracted. As shown in Figure 5A,B, which shows relative viable cell number, only the Δ S2LF had a significant

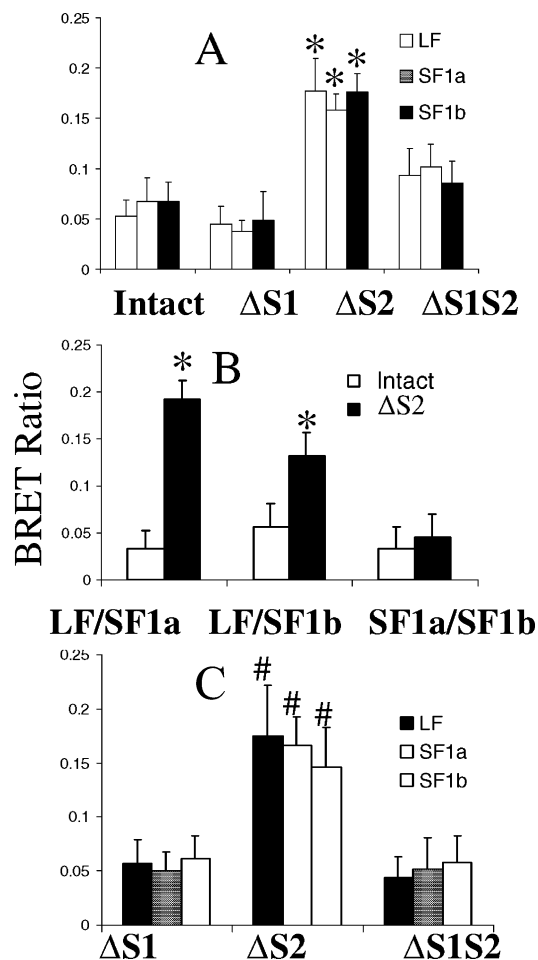


FIGURE 4: Bioluminescence resonance energy transfer with intact and variously deleted receptors and combinations of receptors in the absence (A and B) and presence (C) of PRL. HEK293 cells were transfected with equal quantities of GFP²⁻ or luc-tagged receptors and 48 h later were subjected to BRET analysis initiated by the addition of substrate. For panel B, the same result was obtained regardless of which receptor in the pair was tagged with GFP or luc. Results in all three panels are the mean \pm SEM derived from experiments in which all variables from all panels were concurrently analyzed. Energy transfer is given as the BRET ratio (Emission_{500nm–520nm} – background_{500nm–520nm})/(Emission_{385nm–420nm} – background_{385nm–420nm}). BRET signals obtained from nontransfected cells were considered background in this equation. *, significantly different from the intact version with $p < 0.05$; #, significantly different from ΔS1 and ΔS1S2 ($p < 0.05$) and not different from ΔS2 in panel A.

effect, and this was to increase cell number in both human breast cancer cells (A) and human prostate cancer cells (B). The presented data are only normalized to the control transfected cells so that the degree of change can be appreciated and are not adjusted for transfection efficiency in the culture. Adjustment for transfection efficiency would approximately triple the response. Addition of an antibody to human PRL did not change this overall result (data not shown), although cell number in all incubations went down. The reduced cell number demonstrates that the antibody was effective at binding and neutralizing autocrine PRL. The antibody used was a chicken anti-human PRL raised in the laboratory and demonstrated at the same concentration (1 μ g/mL) to completely block human PRL-stimulated Nb2 cell proliferation during a 3-day incubation (data not shown). The cell proliferation result was therefore unlikely due to autocrine PRL and residual binding to the ΔS2LF. To test the

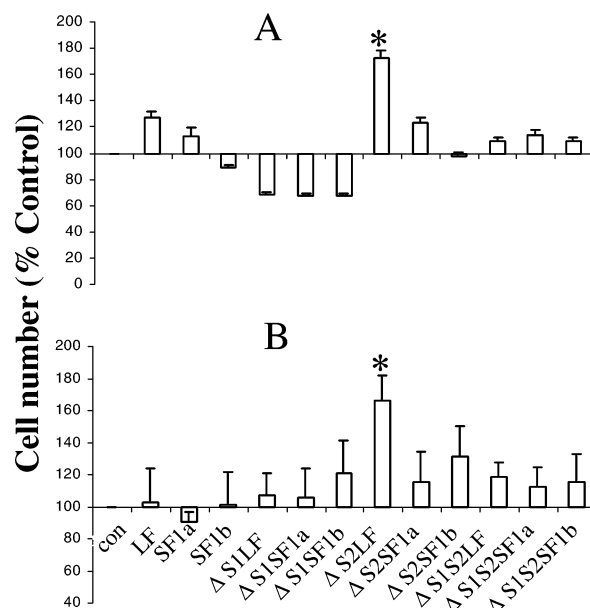


FIGURE 5: Effect of intact and deleted PRLRs on the growth of human breast cancer T47D cells (A) and human prostate cancer DU145 cells (B) in the absence of added PRL. Receptor constructs were transfected into either T47D or DU145 cells. Forty-eight hours post-transfection, the medium with serum was refreshed. After a further 24 h, the medium was changed to serum-free DMEM to conduct the MTS assay. Data are expressed as a percent of the control to illustrate the change induced. The data are not corrected for either transfection efficiency or expression efficiency for reasons discussed in the text. The same overall result was obtained when anti-PRL was added to the incubation, although cell number was decreased in each incubation indicative of an effect of the antibody on autocrine PRL. *, significantly different from the intact counterpart with $p < 0.05$.

possibility that overcrowding of receptors (due to overexpression) could produce proliferation, intact versions of the receptors were also used. No significant effect on cell proliferation (versus controls similarly incubated) was observed with the intact receptors (which were expressed at very similar levels) in the absence of added ligand and presence of anti-PRL. This control also negates the possibility that autocrine PRL may have interacted with the ΔS2 receptors in an intracellular compartment not accessed by anti-PRL.

Also, in human breast cancer cells, but not in the DU145 cells, a consistent reduction, but not a statistically significant decrease, in relative cell number was observed with all three ΔS1 receptors (ΔS1LF, ΔS1SF1a, and ΔS1SF1b).

When endogenous β -casein gene expression was analyzed, a significant increase in expression occurred in ΔS2LF and ΔS2SF1a transfected cells (Figure 6). Once again, although normalized to the control transfected cells, these data have not been normalized for transfection efficiency. This result was also not altered by the addition of anti-human PRL to the medium (data not shown). Since the ΔS2SF1a generated a response that was indistinguishable from that of the ΔS2LF in terms of β -casein gene expression, it seems unlikely that the lack of effect of the ΔS2SF1a on cell proliferation was due to the lower level of expression of this form. Clearly, since the ΔS2SF1b was expressed the most efficiently, the level of expression is not the cause for an absence of significant effect of this form.

Expression of Naturally Occurring ΔS2 PRLRs in Human Cells. Given the constitutive activity of the ΔS2

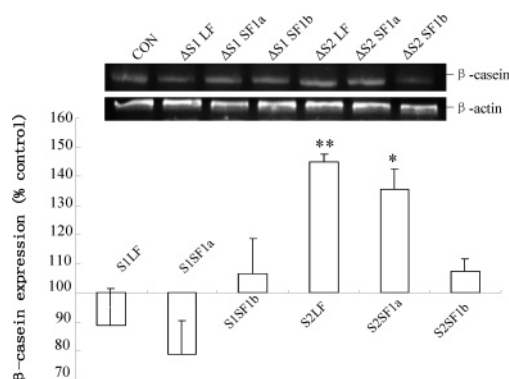


FIGURE 6: Effect of the $\Delta S1$ and $\Delta S2$ receptors on endogenous β -casein gene expression in T47D cells. Forty-eight hours post-transfection, the cells were subjected to a further 24-h incubation in the absence of added PRL and then RT-PCR for β -casein was performed. Data are expressed relative to expression in nontransfected cells (which was the same as that in cells expressing the doubly deleted S1S2 receptor) to illustrate the changes observed. The data are not corrected for either transfection or expression efficiency. The same overall result was obtained in the presence of anti-PRL. *, significantly different from both the nontransfected and $\Delta S1S2$ -transfected cells with $p < 0.05$.

PRLRs, we examined the possibility that some form of this variant occurred naturally in human cells. RT-PCR results showed that in addition to the intact receptors, an amplicon approximately 300 bp shorter than the intact receptor was detected in human prostate cancer cells, LNCaP, DU145, PC-3, and human microvascular endothelial cells, HmVEC-1 (Figure 7A). In DU145 cells, this amplicon was present in larger amounts than the intact receptor. DNA sequence analysis revealed a 303 bp deletion, which generated a transcript lacking the S2 region (amino acids 106–206) and which serendipitously exactly matched the one we had constructed. Next, we examined whether the LF, SF1a and SF1b, each have a $\Delta S2$ variant. Isoform-specific reverse primers were utilized in RT-PCR analysis. Figure 7B shows PC-3 cells as an example and illustrates that all three $\Delta S2$ PRLR isoforms, as well as the intact receptors, can be detected. Once again, sequencing verified the nature of the band. It should be noted when looking at these results that different numbers of cycles were used for each form (see Figure 7 caption). Previous analysis of DU145 and PC3 cells had not detected any intact SF1a (22), but a very small amount of intact and more $\Delta S2SF1a$ was detected in the current study, perhaps due to the use of different primers (see Experimental Procedures). To ensure that the mRNA was translated into protein, Western blot analysis was performed. Figure 7C demonstrates an anti-PRLR positive band in LNCaP, PC3, and T47D cells that runs at the same mol wt (filled arrow) as $\Delta S2SF1b$ in stably transfected T47D cells (TAS2). Because this methodology is not capable of distinguishing between $\Delta S1$ and $\Delta S2$, the possibility that this could be a $\Delta S1$ form was examined by performing RT-PCR with primers specific for such forms. No $\Delta S1$ forms were detected in these cells, thereby supporting the idea that the positive band on the Western was indeed the $\Delta S2SF1b$. The band for a LF is also indicated by the open arrow since this serves as a positive control for the immunoprecipitation and Western. Although the antibody used for both immunoprecipitation and Western recognizes the remaining ECD with the $\Delta S2$ versions, there is no means to quantify the

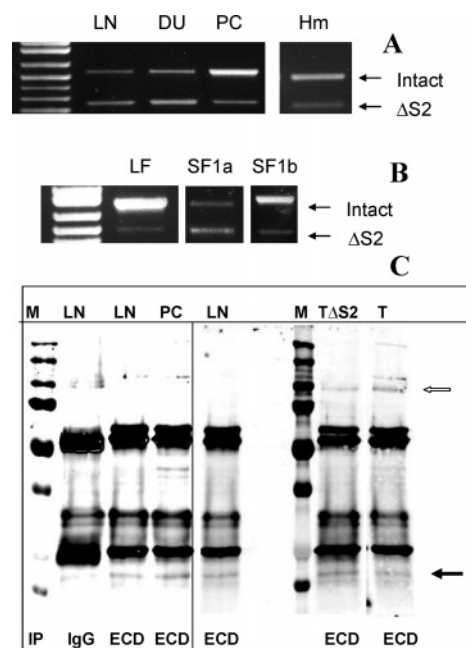


FIGURE 7: Natural expression of $\Delta S2$ PRLRs. (A) Amplicons resulting from the expression of both intact and $\Delta S2$ PRLR were observed in human LNCaP (LN), DU145 (DU), PC3 (PC), and microvessel human endothelial (Hm) cells, and their identities were confirmed by sequencing. (B) Use of form-specific reverse primers demonstrated the presence of a $\Delta S2$ variant of each receptor in all cells, and PC3 cells are illustrated by way of example. Again, the identities of the amplicons were verified by sequencing. Note that different numbers of cycles were required to demonstrate this for each receptor isoform, and thus the panels cannot be compared in terms of relative abundance (LF and SF1a, 35 cycles; SF1b, 40 cycles). (C) After immunoprecipitation, $\Delta S2$ versions of SF1b are illustrated (closed arrow) as detected by Western blot (using the same anti-ECD antibody) in PC3, LNCaP, and T47D (T) cells. Also illustrated is comigration of immunoprecipitated $\Delta S2SF1b$ stably expressed under the control of tetracycline in T47D cells (TAS2). M, molecular mass markers; IP, immunoprecipitated with control isotype-matched antibody (IgG) or antibody against the ECD; open arrow marks a LF of the receptor. The vertical line separates samples run on different gels. The sample from normal T47D cells (T) was run on the same gel, but the lane was cut and pasted to be next to the TAS2 cells to eliminate distracting additional experiments.

relative affinity of this antibody versus the intact ECD and so one cannot draw conclusions as to the relative amounts of each form of the receptor expressed at the protein level. Making the assumption that the mRNA is translated in proportion to its presence, however, it appears that the $\Delta S2$ versions could play a significant role in PRLR function in these cells.

DISCUSSION

To ask the kinds of questions we wanted in this and previous studies (8, 9), a spacer was placed between the C-termini of the receptors and the Rluc or GFP² such that minimal BRET occurred in the intact receptors in the absence of ligand. A conformational change in the intracellular regions of the receptors therefore has to occur to generate a BRET signal. A BRET signal occurs when the donor and acceptor molecules are within 100 Å of each other, such as is necessary to generate transphosphorylation of the receptor-associated kinases (23). This methodology is in contrast to work from the Dufau lab (15) examining PRLRs where no

spacer was reported, resulting in constitutive BRET in intact receptors in the absence of ligand. Our results therefore in no way contradict the notion of receptor predimerization.

The nature of the conformational change resulting in closer approximation of the intracellular regions seems likely to involve rotation of one receptor in relation to the other. We cartooned this for the PRLR in a review in 2005 (24) based in large part on a pioneering study by Seubert et al. on the erythropoietin receptor (25). More recently, work from the Waters' lab on the growth hormone receptor (GHR) (17) supports this concept. In the current study, deletion of the S2 domain of all three receptors resulted in close approximation of the intracellular domains and therefore constitutive BRET for all homopairs and LF-SF heteropairs. Thus, one might suggest that removal of the S2 region allowed a conformation closer to the ligand-bound state, and hence constitutive activation.

Heteropairs between the two Δ S2 SFs did not produce constitutive BRET, a finding in keeping with our previous publication, which found no ligand-induced BRET after this pairing using the intact receptors (8). Thus, removal of the S2 region only facilitates the correct intracellular conformational change with normal receptor pairs and does not allow abnormal pairing. These results also suggest that the S2 region serves a similar function in all three forms of the human PRLR. That function appears to be to hold the intracellular region of the dimerized receptor in a conformation that does not allow signaling until PRL binds.

When BRET was examined using the similarly sized Δ S1 receptors or the more completely deleted Δ S1S2 receptors, there was no significant difference from the controls. This means that the generation of a BRET signal is not the result of reduced bulk of the extracellular region of the receptor, but within the parameters tested in the current study is specific to the changes induced by removal of the S2 region. Since the entire membrane proximal region of S2 was removed, we can also conclude that adoption of an active signaling conformation of the intracellular domain does not require this part, but rather that some part of this subdomain of the intact receptor inhibits the adoption of the active signaling conformation by the intracellular domain, an inhibition that is relieved by the binding of PRL. This result is consistent with previous work by Gourdou et al. using the LF of the rabbit receptor (12). Given that bulk of the extracellular region was not the significant parameter, we can conclude that it is conformation of the ECD that is important and, furthermore, that this constitutes evidence that a change in conformation of the ECD can result in a change in conformation of the intracellular signaling regions of the complex. In the T47D cells, there appeared to be a trend toward decreased cell number with the Δ S1 receptors. This was not reproduced in the DU145 cells. Since this effect was not statistically significant, it may only reflect a chance result. Alternatively, it may be an effect seen only in cells that normally express high levels of intact receptors. Analysis of this possibility will require further experimentation with adenoviral vectors to increase transfection efficiency.

Because there are many unknowns about signal generation from the short receptors, we chose to monitor known end results of signaling, rather than activation of signaling molecules, to link the generation of a BRET signal to a

biologically relevant effect such as cell proliferation and activation of β -casein gene expression.

Of the Δ S2 constitutively active forms, only Δ S2LF increased cell number over that seen with intact receptors in the absence of ligand. Use of the intact receptors was an important control because of the possibility that overexpression by itself could activate signaling. The magnitude of the increase was not large, primarily because these were transient transfection assays with about 30% transfection efficiency. The data presented were not normalized for transfection efficiency, but had they been they would have shown more than a doubling in the actual transfected cells. The same result was obtained with both T47D cells and DU145 cells which inherently express very different levels of PRLRs. From the current results, therefore, it is clear that signaling through the LF increases cell number. This function for the LF is in agreement with the results of Lee et al. (13) and work showing activation of cyclin D1 by the LF (26). Analysis of β -casein expression showed that constitutive activation of both the LF and SF1a increased expression, a result consistent with many previous studies for the LF and with a previous study from this laboratory in collaboration with the Vonderhaar group on the SF1a (8). Once again, the data presented were not normalized for transfection efficiency, and hence the effect per transfected cell is greater than illustrated. It is also interesting to note that the effect of Δ S2SF1a is very similar to that of LF, despite the fact that Δ S2SF1a is expressed at a lower overall level in the cells. This may reflect a greater efficiency of SF1a receptors or may reflect an equal appropriate localization in the cell. Certainly, with the intact receptors, there was very similar cell-associated PRL-Gluc despite very different overall levels of expression. As described previously (8), there is no suggestion that activation of β -casein does not require Stat 5 since these cells have a high background Stat 5 activation to which activation of other signaling cascades can add.

On the basis of these findings, one might suggest that PRL promotes proliferation and differentiation in cells expressing mostly LF and has a greater differentiative effect in cells with a significant expression of SF1a. Thus, the deduced role for prolactin in cancer cell lines may be cell line dependent. The current experiments shed no light on the role of the SF1b receptor since constitutive activity of this receptor did not significantly alter either basal cell proliferation or β -casein expression during the duration of these transient transfection experiments.

In presenting the data, we have chosen not to normalize to either transfection efficiency or the level of expression, although we have reported their similarities or differences throughout. We opted not to normalize to transfection efficiency since this was not reflective of expression, but only the percentage of cells transfected. To additionally normalize to expression gave us two concerns. The first was that the data would then be triply normalized, and the second was whether this was appropriate since although total expression of, for example, LF and SF1a was rather different, the amount of cell-associated PRL-Gluc with both intact receptors was the same. This suggests that the total number of intact receptors on the plasma membrane was the same and that this might be the meaningful parameter to which to normalize. That said, however, it is unclear whether the Δ S2 receptors need a plasma membrane localization since they

do not require ligand and, at least in other members of this cytokine family (27), Janus kinase 2 can associate with the receptor in the rough endoplasmic reticulum. With these unknowns, it seemed more appropriate to present the data in a more raw form and discuss the possible role of differential expression as we have.

The tertiary structure of the extracellular domain of the rat receptor, as determined by crystallization with placental lactogen, shows that the S1 and S2 regions form six ligand-binding loops (L1–L6). Of these, L1, L2, and L3 are located in S1, and L5 and L6 are located in S2, while L4 is in the linker region between the domains (28). Since the Δ S1 and Δ S2 receptors retained some of the loops, it was not unreasonable to ask whether there was any residual ligand binding for these forms. Ligand binding was assessed using a new fusion protein developed in this laboratory in which the C-terminus of PRL is linked to *Gaussia* luciferase via a 14 amino acid linker. With this size linker and expression in eukaryotic cells, both the luciferase and PRL are biologically active. The luciferase makes the assay very sensitive, allowing us to look at binding under more physiological circumstances. Using an amount of the PRL-Gluc equivalent to 50 ng/mL unmodified PRL, we were able to show similar levels of cell-associated luciferase activity for each of the intact receptors. Removal of S1 or S2 reduced cell-associated luciferase activity to about 25% of that with the intact receptors, a result that was clearly statistically significant. Not significant, however, was the difference between residual luciferase activity and the subtracted nonspecific activity (activity after binding in the presence of a 50-fold excess of unmodified PRL). HEK293 cells do not produce PRL, and hence the constitutive BRET seen with the Δ S2 receptors cannot be the result of PRL binding to the remaining portion of the receptor. When PRL was added to the system, there was also no change in the amount of BRET. DU145 and T47D cells, on the other hand, produce autocrine PRL (29, 30) and so the potential existed for this autocrine PRL to affect proliferation or β -casein expression through some residual binding. However, when antibody was added to the medium during these analyses, there was no change in the overall result, although cell number in each incubation was decreased, thereby demonstrating that the antibody bound and neutralized the autocrine PRL. Thus, even though others have reported residual binding for a LF devoid of most of the S1 region (31), our results show that this is insufficient to produce BRET or effects on cell proliferation or endogenous β -casein expression. However, in this cited article the very N terminal S1 residues were removed, whereas in our case, amino acids 1–3 and 103–106 remained in our Δ S1 constructs. Perhaps these amino acids before the S2 region decreased access for binding. Our Δ S1 and Δ S2 constructs were very similar to those of Gourdou et al. (12) who found no residual binding for either. For the purposes of the current study, the most important issue, substantiated by the lack of effect of (1) intact receptors in the absence of added ligand and presence of anti-PRL, (2) added PRL on the BRET analysis, and (3) anti-PRL on the overall proliferation and β -casein result, was constitutive activity of the Δ S2 receptors.

Given the potential clinical importance of constitutively active receptors, we also asked whether there was any evidence for naturally occurring constitutively active Δ S2 receptors in human cells. Analysis by RT-PCR showed the

expression (at least at the level of mRNA) of Δ S2 forms in three prostate cancer cell lines and human microvascular endothelial cells. The high level of expression of other PRLR forms in T47D cells made definitive PCR demonstration of Δ S2 forms difficult, and therefore these data are not shown. More detailed analysis in the prostate cancer cells showed that the Δ S2 version of all three receptor isoforms could be detected. Not only were these seen as PCR amplicons, but also the bands were sequenced to confirm their identity. In addition, Western analysis of PC3 LNCaP and T47D cells showed an anti-PRLR positive band equivalent in size to transfected Δ S2SF1b. When the natural Δ S2 versions of all three receptors were sequenced, they serendipitously were found to have exactly the same sequence as those we had constructed. It therefore appears that human cells have the capability of splicing the receptor to produce a constitutively active form. Of importance is the number of cell types shown to express the Δ S2 forms of the receptor (LNCaP, PC3, DU145, T47D, and HmVEC) since this makes the forms less likely to be an oddity of a single transformed cell line. The splice site is not a common one but has similarities to other reported sites (32). This raises the possibility that the Δ S2 varieties play roles in the regulation of cell proliferation and differentiation.

We conclude that a change in the conformation of the extracellular domain can indeed cause a change in the conformation of the intracellular signaling region, thereby supporting the as yet unproven concept that ligand binding to the ECD of predimerized receptors could have a similar effect. Using the constitutively active receptors, we also directly compared the functions of the LF and both SFs in the regulation of cell proliferation and β -casein expression and found that constitutive activation of the LF results in increased cell number, whereas constitutive activation of either the LF or SF1a results in increased β -casein gene expression. In addition, we showed that human cells express Δ S2 versions of the receptors, in some cells at levels greater than the intact versions at the mRNA level, suggesting a possible role in the regulation of proliferation and differentiation.

ACKNOWLEDGMENT

We are grateful to Dr. Barbara K. Vonderhaar for providing the original receptor constructs and to Dr. David Johnson for allowing us continued access to the spectrofluorimeter.

REFERENCES

1. Nevalainen, M. T., Valve, E. M., Ingleton, P. M., Nurmi, M., Martikainen, P. M., and Harkonen, P. L. (1997) Prolactin and prolactin receptors are expressed and functioning in human prostate. *J. Clin. Invest.* 99, 618–627.
2. Neville, M. C., McFadden, T. B., and Forsyth, I. (2002) Hormonal regulation of mammary differentiation and milk secretion. *J. Mammary Gland Biol. Neoplasia* 7, 49–66.
3. Goffin, V., Binart, N., Touraine, P., and Kelly, A. P. (2002) Prolactin: the new biology of an old hormone. *Annu. Rev. Physiol.* 64, 47–67.
4. Hu, Z., Zhuang, L., Maria, L., and Dufau, M. L. (1996) Multiple and tissue-specific promoter control of gonadal and non-gonadal prolactin receptor gene expression. *J. Biol. Chem.* 271, 10242–10246.
5. Nagano, M., and Kelly, P. A. (1994) Tissue distribution and regulation of rat prolactin receptor gene expression. Quantitative analysis by polymerase chain reaction. *J. Biol. Chem.* 269, 13337–13345.

6. Cosman, D. (1993) The hematopoietin receptor superfamily. *Cytokine* 5, 95–106.
7. Bole-Feysot, C., Goffin, V., Edery, M., Binart, N., and Kelly, P. A. (1998) Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocr. Rev.* 19, 225–268.
8. Tan, D., Johnson, D. A., Wu, W., Zeng, L., Chen, Y. H., Chen, W. Y., Vonderhaar, B. K., and Walker, A. M. (2005) Unmodified prolactin (PRL) and S179D PRL-initiated bioluminescence resonance energy transfer between homo- and hetero-pairs of long and short human PRL receptors in living human cells. *Mol. Endocrinol.* 19, 1291–1303.
9. Langenheim, J. F., Tan, D., Walker, A. M., and Chen, W. Y. (2005) Two wrongs can make a right: dimers of prolactin and growth hormone receptor antagonists behave as agonists. *Mol. Endocrinol.* 19, 1291–1303.
10. Goffin, V., and Kelly, P. A. (1997) The prolactin/growth hormone receptor family: structure/ function relationships. *J. Mammary Gland Biol. Neoplasia* 2, 7–17.
11. Christinger, H. W., Elkins, P. A., Sandowski, Y., Sakal, E., Gertler, A., Kossiakoff, A. A., and de Vos, A. M. (1998) Crystallization of ovine placental lactogen in a 1:2 complex with the extracellular domain of the rat prolactin receptor. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* 54, 1408–1411.
12. Gourdou, I., Gabou, L., Paly, J. A., Kermabon, A. Y., Belair, L., and Djiane, J. (1996) Development of a constitutively active mutant form of the prolactin receptor, a member of the cytokine receptor family. *Mol. Endocrinol.* 10, 45–56.
13. Lee, R. C., Walters, J. A., Reyland, M. E., and Anderson, S. M. (1999) Constitutive activation of the prolactin receptor results in the induction of growth factor-independent proliferation and constitutive activation of signaling molecules. *J. Biol. Chem.* 274, 10024–10034.
14. Sakal, E., Elberg, G., and Gertler, A. (1997) Direct evidence that lactogenic hormones induce homodimerization of membrane-anchored prolactin receptor in intact Nb2–11C rat lymphoma cells. *FEBS Lett.* 410, 289–292.
15. Qazi, A. M., Tsai-Morris, C. H., and Dufau, M. L. (2006) Ligand-independent homo- and heterodimerization of human prolactin receptor variants: inhibitory action of the short forms by heterodimerization. *Mol. Endocrinol.* 20, 1912–1923.
16. Gadd, S. L., and Clevenger, C. V. (2006) Ligand-independent dimerization of the human prolactin receptor isoforms: functional implications. *Mol. Endocrinol.* 20, 2734–2746.
17. Brown, R. J., Adams, J. J., Pelekanos, R. A., Wan, Y., McKinstry, W. J., Palethorpe, K., Seeber, R. M., Monks, T. A., Eidne, K. A., Parker, M. W., and Waters, M. J. (2005) Model for growth hormone receptor activation based on subunit rotation within a receptor dimer. *Nat. Struct. Mol. Biol.* 12, 814–821.
18. Constantinescu, S. N., Keren, T., Socolovsky, M., Nam, H., Henis, Y. I., and Lodish, H. F. (2001) Ligand-independent oligomerization of cell-surface erythropoietin receptor is mediated by the transmembrane domain. *Proc. Natl. Acad. Sci. U.S.A.* 98, 4379–4384.
19. Chen, T. J., Kuo, C. B., Tsai, K. F., Liu, J. W., Chen, D. Y., and Walker, A. M. (1998) Development of recombinant human prolactin receptor antagonists by molecular mimicry of the phosphorylated hormone. *Endocrinology* 139, 609–616.
20. Huang, K. T., Chen, Y. H., and Walker, A. M. (2004) Inaccuracies in MTS assays: major distorting effects of medium, serum albumin, and fatty acids. *BioTechniques* 37, 406–412.
21. Wu, W., Ginsburg, E., Vonderhaar, B. K., and Walker, A. M. (2005) S179D prolactin increases vitamin D receptor and p21 through upregulation of short 1b prolactin receptor in human prostate cancer cells. *Cancer Res.* 65, 7509–7515.
22. Rozakis-Adcock, M., and Kelly, P. A. (1992) Identification of ligand binding determinants of the prolactin receptor. *J. Biol. Chem.* 267, 7428–7433.
23. Watson, C. J., and Burdon, T. G. (1996) Prolactin signal transduction mechanisms in the mammary gland: the role of the Jak/Stat pathway. *Rev. Reprod.* 1, 1–5.
24. Walker, A. M. (2005) Prolactin receptor antagonists. *Curr. Opin. Invest. Drugs* 6, 378–385.
25. Seubert, N., Royer, Y., Staerk, J., Kubatzky, K. F., Moucadel, V., Krishnakumar, S., Smith, S. O., and Constantinescu, S. N. (2003) Active and inactive orientations of the transmembrane and cytosolic domains of the erythropoietin receptor dimer. *Mol. Cell* 12, 1239–1250.
26. Brockman, J. L., and Schuler, L. A. (2005) Prolactin signals via Stat5 and Oct-1 to the proximal cyclin D1 promoter. *Mol. Cell. Endocrinol.* 239, 45–53.
27. Huang, L. J., Constantinescu, S. N., and Lodish, H. E. (2001) The N-terminal domain of Janus kinase 2 is required for Golgi processing and cell surface expression of erythropoietin receptor. *Mol. Cell* 8, 1327–1338.
28. Elkins, P. A., Christinger, H. W., Sandowski, Y., Sakal, E., Gertler, A., de Vos, A. M., and Kossiakoff, A. A. (2000) Ternary complex between placental lactogen and the extracellular domain of the prolactin receptor. *Nat. Struct. Biol.* 7, 808–815.
29. Xu, X., Kreye, E., Kuo, C. B., and Walker, A. M. (2001) A molecular mimic of phosphorylated prolactin markedly reduced tumor incidence and size when DU145 human prostate cancer cells were grown in nude mice. *Cancer Res.* 61, 6098–6114.
30. Ginsburg, E., and Vonderhaar, B. K. (1995) Prolactin synthesis and secretion by human breast cancer cells. *Cancer Res.* 55, 2591–2595.
31. Kline, J. B., Ryczyn, M. A., and Clevenger, C. V. (2002) Characterization of a novel and functional human prolactin receptor isoform (delta S1PRLr) containing only one extracellular fibronectin-like domain. *Mol. Endocrinol.* 16, 2310–2322.
32. Jackson, I. J. (1991) A reappraisal of non-consensus mRNA splice sites. *Nucleic Acids Res.* 19, 3795–3798.

BI7013882

Prolactin and estradiol utilize distinct mechanisms to increase serine-118 phosphorylation and decrease levels of estrogen receptor α in T47D breast cancer cells

YenHao Chen · KuangTzu Huang ·
KuanHui E. Chen · Ameae M. Walker

Received: 5 January 2009 / Accepted: 6 April 2009
© Springer Science+Business Media, LLC. 2009

Abstract Potential interactions between prolactin (PRL) and estradiol (E2) in breast cancer cells were explored by examining the effect of PRL on estrogen receptor (ER) serine-118 phosphorylation, ER down-regulation, and E2-stimulated cell proliferation. Both E2 and PRL resulted in prolonged ER α serine-118 phosphorylation, but used different signaling pathways to achieve this end. Both hormones also decreased the amount of ER α , but the mechanisms were different: for E2, the decrease was rapid and resulted from proteasomic degradation, whereas for PRL the decrease was slow and resulted from an effect on levels of ER α mRNA. PRL alone had no effect on cell number, but enhanced the increase in number in response to E2. These results are the first to demonstrate similar effects of PRL and E2 on parameters considered key to E2's effects. This suggests heretofore unrecognized and potentially important interactions between these two hormones in the natural history of breast cancer.

Keywords Breast cancer · Estrogen receptor · Prolactin · Crosstalk

Introduction

Both estradiol (E2) and prolactin (PRL) are absolutely required for full development and function of the mammary gland, with effects of each on both cell proliferation and differentiation. Evidence also suggests that exposure to increased levels of these hormones increases the risk for

breast cancer [1–5]. However, relatively little is known about how E2 and PRL interact at the level of mammary epithelium.

The two major forms of the estrogen receptor, ER α and ER β , are produced by different genes [1]. Both belong to the steroid/nuclear receptor superfamily of ligand-regulatable transcription factors [6,7] and have similar functional domains. These include an N-terminal domain, a central DNA-binding domain, and a C-terminal ligand-binding domain. When the receptor binds E2, a conformational change occurs. This is followed by translocation to the nucleus, recruitment of co-factors, binding to a target gene and regulation of gene transcription [8, 9]. In addition to this genomic effect, responses can occur within minutes after E2 administration. E2 has for example been reported to rapidly activate MAP kinase or Akt in several cell lines, including breast cancer cell lines [10–12].

Phosphorylation of serine residues is important for ER-mediated transcription and probably the most important site in ER α is serine-118 [13–21]. Here, phosphorylation has been reported to result from the activity of MAP kinase, PI3-kinase/Akt, and GSK3 activation [1, 15, 18–22]. In addition to phosphorylation by E2, epidermal growth factor and thyroid hormone can also cause transient serine-118 phosphorylation of the ER via activation of MAP kinase in breast cancer cells [1].

E2 also modulates the level of ER protein in a complex way that balances feedback down-regulation with preservation of a prolonged response to E2 if levels of the hormone remain high [3, 21]. The half life of the ER is only 3–4 h in the presence of ligand, but ~5 days in the absence of ligand [21, 23, 24]. ER protein is degraded in a hormone-dependent manner and the proteasome inhibitor, MG132, blocks degradation and promotes accumulation of ERs [25].

Y. Chen · K. Huang · K. E. Chen · A. M. Walker (✉)
Division of Biomedical Sciences, University of California,
Riverside, CA 92521, USA
e-mail: ameae.walker@ucr.edu

PRL is considered to induce most of its biological effects through a receptor present in the plasma membrane. The majority of studies have focused on the robust activation of the Jak2-Stat5 pathway following the binding of PRL to the long form of the PRL receptor (PRLR). Jak2 is constitutively associated with the PRLR [26, 27]. Upon binding PRL, there is a conformational change in pre-formed PRLR dimers that converts inactive to active dimers [28, 29]. This conformational change initiates Jak2 trans-phosphorylation, tyrosine phosphorylation of the PRLR and generation of docking sites for the SH2 domains of Stat5 proteins [30, 31]. Stat5 proteins can be activated by Jak2 and become either homodimers or heterodimers with other transcription factors. These dimers translocate to the nucleus where they regulate gene transcription [32–34]. In addition to the Jak2-Stat5 pathway, PRL also activates MAP kinase [30, 35–37], PKC δ [38], c-Src [39–43] and PI3-kinase [39, 44, 45], as well as activating potassium channels [46].

T47D cells are ER positive and PRLR positive. Both hormones have been individually examined for their effects on breast cancer cells, but few studies have focused on the potential interactions of these two hormones. In the current study, we have examined the effect of PRL on ER α serine-118 phosphorylation, ER down-regulation, and E2-stimulated cell proliferation. We demonstrate that both PRL and E2 stimulation result in ER α serine-118 phosphorylation and down-regulation, but that the mechanisms used to achieve these ends, at least within the first 24 h, are distinct.

Materials and methods

Cell culture

T47D cells (American Type Culture Collection, Rockville, MD) were routinely cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin and 100 μ g/ml streptomycin (Invitrogen) at 37°C in 5% CO₂ in a humidified incubator. Semi-confluent cells in 60 mm dishes were used for most experiments. Prior to all experiments, the growth medium was changed to phenol red-free RPMI containing 5% charcoal-stripped horse serum (CoCalico Biologicals, Reamstown, PA) overnight. Cell treatments are given in the figure legends.

Hormones and inhibitors

Human PRL was produced as described previously [47]. E2 from Sigma chemical company (St Louis, MO), was dissolved in ethanol and used from a 1,000 fold stock. The

proteasome inhibitor, MG132, and the MEK inhibitor, PD98059, were from EMD Biosciences (La Jolla, CA). The PI3-kinase inhibitors, LY204002 and wortmannin were from Alexis Biochemicals (San Diego, CA) and Calbiochem (La Jolla, CA), respectively. Diluents served as controls.

Western blot

For most blots, 40 μ g protein from whole cell lysates were loaded per lane on reducing SDS gels. After electrophoresis, protein was transferred to nitrocellulose and the membrane was blocked with 5% non-fat milk in phosphate buffered saline containing 0.1% Tween 20. Membranes were incubated with primary antibodies—rabbit polyclonal anti-ER α (H-184), ER β (H-150), ERK1, and actin (1:1,000, Santa Cruz Biotechnology, Inc, Santa Cruz, CA), Akt, phospho-Akt, and serine-118 ER α (1:1,000, Upstate, Charlottesville, VA), or mouse monoclonal anti-phospho ERK1/2 (1:1,000, Upstate) for 1 h at room temperature or overnight at 4°C. After 6 washes for a total of 30 min, the membranes were incubated in either goat anti-rabbit or goat anti-mouse IgG conjugated with horseradish peroxidase, as appropriate, for 1 h at room temperature (1:10,000). After a further wash period, labeled proteins were detected using the enhanced chemiluminescent system (Amersham Biosciences, Piscataway, NJ). Membranes were stripped prior to re-probing in RestoreTM stripping buffer (Pierce Biotechnology Inc., Rockford, IL). For the blots used for semi-quantitative analysis, all steps in the process were determined to be within the linear range so that densities on the final autoradiogram could be quantified. This included monitoring complete transfer to the membrane, titration of antibodies, incubation time and film exposure. Test runs with prolonged stripping of reprobed membranes were used to ensure complete antibody stripping to establish that residual labeling with the first antibody pair did not interfere with the second antibody pair used. For illustration purposes, we have presented high contrast images of the blots.

Real time RT-PCR

Total RNA was extracted with TRIzolTM (Invitrogen) and 5 μ g was used for reverse transcription using M-MLV reverse transcriptase and oligo dT (Promega, Madison, WI). Primer sequences used to amplify ER α were as follows: TATGATCCTACCAGACCCTTCA (forward) and TCAGACTGTGGCAGGGAAAC (reverse). The house-keeping gene, β -actin, was used for normalization. For this, primers were AAAGACCTGTACGCCAACAC (forward) and GTCATACTCCTGCTTGCTGAT (reverse). SYBR green-based technology was employed for real time PCR

using the ABI Prism SDS 7,700 sequence detection system (Applied Biosystems, Foster City, CA). Power SYBR green Mastermix (12.5 µl) containing SYBR green 1 dye, AmpliTaq Gold DNA polymerase, dNTPs containing a mixture of dUTP and dTTP, ROX passive reference dye and buffer was mixed, along with 1 µl each of the sense and anti-sense primers (10 mM each) for each gene of interest, 2.5 µl of the template (50 ng/ml), and 8.0 µl of double distilled H₂O. All samples were run simultaneously and in triplicate on the same 96-well optical microtiter plate. The following amplification parameters were used: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A separate melting curve analysis was also run to characterize the fluorescence intensities of the amplified products and to verify the absence of contamination. A relative quantification method was utilized to calculate the fold difference in the expression of each gene in the treatment groups, using the following equation: $\Delta\Delta C_t = 2^{-(\text{experimental } \Delta C_t - \text{control } \Delta C_t)}$, where C_t is the threshold cycle.

Assay of viable cell number

Cells were seeded at 5×10^3 cells per well of 96 well plates and pre-treated as for all other experiments. For the experiments involving inhibitors, these or their diluents were added 2 h before hormones and viable cell number was measured at the end of the 3-day incubation in hormones. After changing medium to Dulbecco's phosphate buffered saline containing 4.5 g/L glucose [48], cell number was assessed using an assay based on the reduction of the soluble tetrazolium salt: 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS assay, Promega), according to the manufacturer's instructions.

Statistical analysis

All results were representative of at least three experiments. For the quantitative results shown in Figs. 5, 6, 7, 8, analysis of significance was by ANOVA with subsequent posttests and corrections for multiple comparisons.

Results

E2 causes serine-118 phosphorylation of ER α and serine-118 phosphorylation has been shown to be important for many of the transcriptional effects of ER α [14–21]. Data presented in Fig. 1 confirm the ability of E2 to cause serine-118 phosphorylation of ER α , specifically in T47D cells and in our hands, and show that continued incubation in E2 resulted in continued phosphorylation of ER α from 15 min

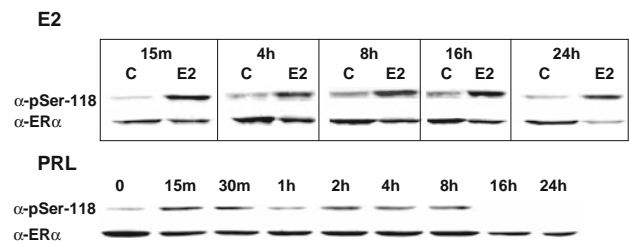


Fig. 1 Effect of PRL and E2 on serine-118 phosphorylation of ER α . T47D cells were pre-incubated in phenol red-free medium with 5% charcoal-stripped gelding serum overnight and then treated with 1 nM E2 or 1 µg/ml PRL in the same medium. Whole cell lysates were collected at the indicated time points. Western blots were performed with ser-118 phosphorylation site-specific anti-ER α (α -pSer-118). The blots were then stripped and re-probed with anti-ER α (α -ER α). The image, which shows results from the same set of cells, is a composite for E2 treatment blots (indicated by boxes), but the control (C) for each time point allows comparison between control and E2 stimulation at each time point. Control treatment was with the diluent for E2, ethanol

to 24 h. Although this is a composite figure, there is a control lane for each time point that allows a qualitative appreciation of continued ER α phosphorylation across all time points. A similar experiment conducted with PRL in the absence of E2 (in phenol red-free medium supplemented with 5% charcoal-stripped horse serum) showed that PRL could also stimulate serine-118 phosphorylation and that this stimulation was also long-lived, although only detected to 8 h in this instance.

Because both hormones resulted in prolonged serine-118 phosphorylation, we next asked whether they utilized similar signaling pathways to achieve this effect. Both the PI3-kinase and MAP kinase pathways have previously been implicated in E2-stimulation of serine-118 phosphorylation [1, 15, 18–22]. We therefore, examined the effect of these two inhibitors on E2-stimulated cells. Figure 2 shows that the PI3-kinase inhibitor, LY294002 was able to reduce serine-118 phosphorylation in response to E2 at 30 min (2b) and 24 h (2c). However, the PI3-kinase inhibitor, wortmannin had no effect (2b). In both instances, levels of p-Akt (the downstream target) were apparently consistent with efficient inhibition of PI3-kinase, but for reasons that will be discussed later the results with wortmannin were considered more reliable. At the 30 min and 24 h time points, Akt was not appreciably activated by E2. Inhibition of the MAP kinase pathway with PD98059 was without effect on serine-118 phosphorylation by E2, despite the fact that PD98059 effectively blocked E2-stimulated activation of ERK1/2. In other words, although E2 may have activated ERK (some activation is more obvious in the left panel of Fig. 2b), there was no evidence that ERK activation was responsible for E2-stimulated serine-118 phosphorylation of ER α .

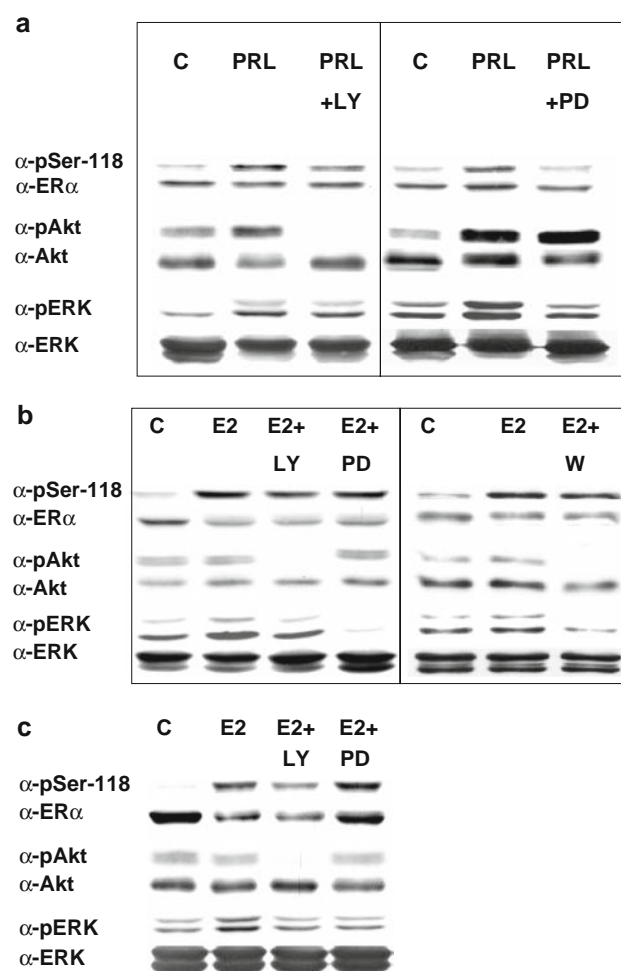


Fig. 2 Effect of kinase inhibitors on E2 and PRL stimulation of serine-118 phosphorylation. T47D cells were pre-incubated in phenol red-free medium with 5% charcoal-stripped gelding serum overnight. Addition of LY294002 (LY) or PD98059 (PD) to a final concentration of 20 μ M, or wortmannin (W) to a final concentration of 1 μ M occurred for 2 h in fresh medium (with the same composition) prior to the addition of hormones. The cells were then treated with E2 (1 nM) or PRL (1 μ g/ml) for 30 min (**a**, **b**) or 24 h (**c**) in the continued presence of the inhibitors. Whole cell lysates were collected and Western blots performed. Triplicate lanes were probed with either phosphorylation site-specific anti-ER α (α -pSer-118), phosphorylation site-specific anti-Akt (α -p-Akt) or phosphorylation site-specific anti-ERK (α -p-ERK), stripped and re-probed for total ER α , Akt or ERK. Panels **a** and **b** are composites from 2 experiments each, both of which have a control for comparison. Controls used diluents (ethanol for E2, DMSO for LY, PD and W)

Similar experiments analyzing the effects of LY294002 and PD98059 on the ability of PRL to stimulate serine-118 phosphorylation of ER α (LY294002 can be considered reliable with PRL stimulation) clearly showed a substantial inhibitory effect of both compounds, once again with concurrent illustration of efficacy on the target kinases. There was at least a large degree of independence of the PI3-kinase and MAP kinase pathways since LY294002 had little effect on ERK phosphorylation by PRL, and PD98059 had very

little if any effect on Akt phosphorylation by PRL. Efficacy of LY294002 is shown by blockade of Akt activation and efficacy of PD98059 is shown by blockade of ERK activation.

A normal result of E2 stimulation is down-regulation of ER α [3, 21, 23–25]. We therefore, examined whether PRL also caused down-regulation of ER α . Figure 3 shows a dose-response to increasing concentrations of E2 or PRL for 24 h. Both hormones result in reduced ER α , the effect is specific to ER α (no effect on ER β), and the response to PRL occurs within the physiological range of 10–100 ng/ml.

The ability of E2 to decrease the amount of ER α has been previously demonstrated to involve proteasomic degradation [25]. We therefore, examined the time course of ER α degradation in response to E2 in the absence and presence of the proteasome inhibitor, MG132. The amount of ER α present in whole cell extracts was reduced by 2 h. This effect of E2 was maintained for the duration of the experiment, which in this case was 16 h (Fig. 4). Other experiments (Fig. 1) suggested maintenance of the effect to 24 h. The ability of E2 to reduce the amount of ER α was completely blocked by the proteasome inhibitor. A similar experiment with PRL showed that it took 16 h for PRL to reduce the amount of ER α and that the reduction, when it occurred, was not blocked by the proteasome inhibitor (Fig. 4).

Since the effect of PRL on the amount of ER α was not via proteasomic degradation and the response to PRL was much slower, an effect on mRNA levels was considered. Figure 5 shows that PRL reduced ER α mRNA by half after

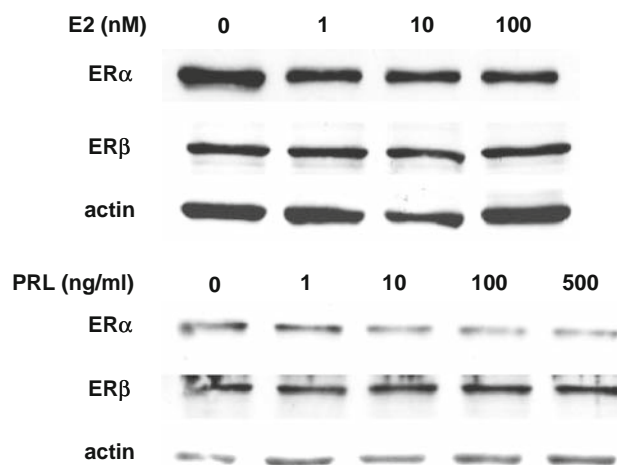


Fig. 3 Specificity and dose dependency of effect of E2 or PRL on ER α . T47D cells were pre-incubated in phenol red-free medium with 5% charcoal-stripped gelding serum overnight and then treated with E2 or PRL at the indicated doses for 24 h in the same medium. Cell lysates were collected and Western blots performed. Blots were probed with anti-ER α (α -ER α), stripped and re-probed with anti-ER β (α -ER β) and then stripped and re-probed with anti-actin

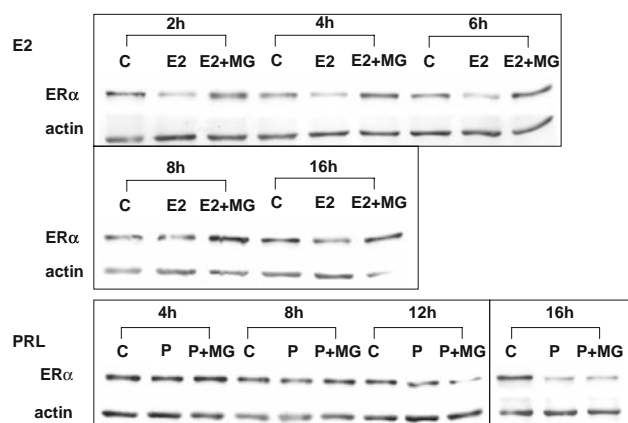


Fig. 4 Effect of the proteasome inhibitor, MG132, on the E2- and PRL-initiated decrease in ER α . T47D cells were pre-incubated in phenol red-free medium with 5% charcoal-stripped gelding serum overnight and then pre-treated for 2 h with or without 10 μ M MG132 (MG) in the same medium. 1 nM E2 or 1 μ g/ml PRL (P) was then added and whole cell lysates were collected at the indicated time points. Western blots were performed. All samples were from the same set of cells. The number of samples necessitated the running of two gels each for E2 and PRL treatment (indicated by *connected boxes*), but all samples were blotted, probed, and exposed together. Other abbreviations, as for previous figures

a 24 h incubation, as measured by real time RT-PCR. Concurrent analysis of the effect of E2 showed no change in this time frame.

Analysis of the signaling pathways leading to this effect of PRL on mRNA levels showed that inhibition of PI3-kinase and MAP kinase partially reversed the effect of PRL (Fig. 6a). Responses to both inhibitors were similar at both the protein and mRNA levels.

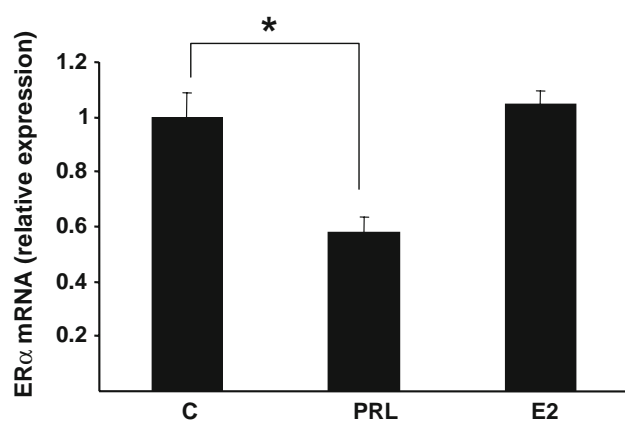


Fig. 5 Effect of PRL and E2 on the relative level of mRNA for ER α . T47D cells were pre-incubated as before and then treated with PRL (1 μ g/ml) or E2 (1 nM) for 24 h in medium with the same composition. RNA was extracted and real time RT-PCR was performed. Data were normalized to β -actin and then the control level was set at 1. The results are presented as the mean \pm SE. * $P < 0.05$

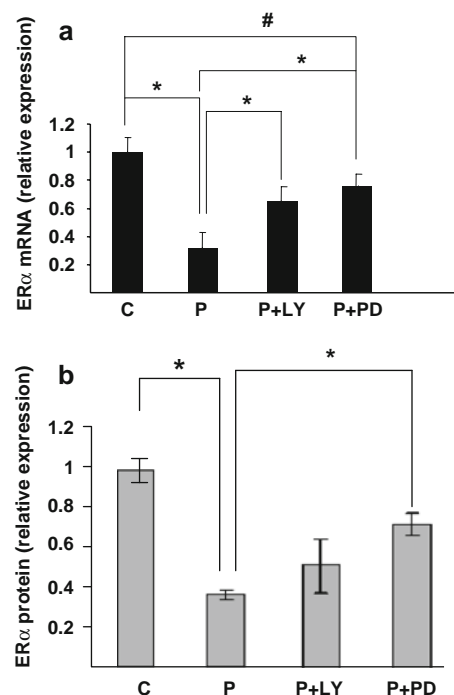


Fig. 6 Ability of kinase inhibitors to reverse the decrease in mRNA and protein levels produced by PRL. T47D cells were pre-incubated as before. For the last 2 h, the inhibitors were added to a final concentration of 20 μ M. PRL was then added for 24 h in the continued presence of the inhibitors. RNA was extracted and real time RT-PCR was performed. Replicate samples were extracted and used for quantitative Western blot analysis. Data were normalized to β -actin and then the control level was set at 1. Results are presented as the mean \pm SE. C control, P PRL, LY LY294002, PD PD98059. * $P < 0.05$; # not different from control

Although E2 increases cell number despite induced down-regulation of ER α , it was unclear how the potentially counter-regulatory results of serine-118 phosphorylation and ER α amount would manifest themselves in response to PRL and then PRL plus E2. We therefore, examined the result of a 3-day incubation of T47D cells in either PRL alone, E2 alone or both hormones together (Fig. 7). In our hands, PRL alone at 1 μ g/ml had no effect on viable cell number, whereas it enhanced the increase in cell number in response to E2 (1 nM). It should be noted when looking at the figure that this experiment was conducted in the E2-depleted medium. Lower concentrations of PRL (100–500 ng/ml) were equally ineffective alone, demonstrating that the lack of effect was not a consequence of a biphasic PRL dose-response (data not shown).

Use of the inhibitors at concentrations without effect on basal cell number (wortmannin at 0.5 μ M and PD98059 at 2 μ M) demonstrated that they had no effect on E2-stimulated proliferation while inhibiting the additional proliferation in response to PRL in combined incubations (Fig. 8).

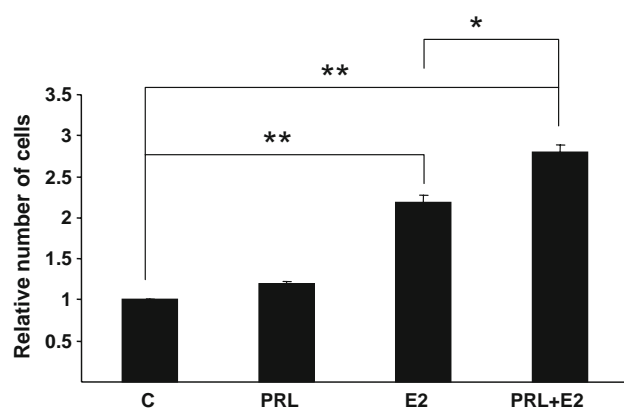


Fig. 7 Effect of PRL alone, and with E2, on viable cell number. T47D cells were pre-incubated as before and then treated with PRL (1 μ g/ml) or E2 (1 nM), or both for 3 days in phenol red-free medium containing 5% charcoal-stripped gelding serum. Relative cell number was determined by MTS assay. The results were normalized to the control to combine experiments and are presented as the mean \pm SE. * $P < 0.05$; ** $P < 0.01$

Discussion

Serine-118 phosphorylation of ER α is important for activation of transcription [14–21] and has been described by

others as producing ligand-independent ER activation of transcription [49, 50]. In addition, a recent study also suggests that it is important for stabilization of ER α [21], ensuring the maintenance of a response to E2 during prolonged exposure to this hormone. PRL caused serine-118 phosphorylation, thereby suggesting possible E2-independent effects. However, this did not translate to an effect on cell number in response to PRL alone. Presumably, without ligand and the accompanying conformational change and/or phosphorylation of additional sites in ER α , not all of the necessary co-factors were recruited to result in increased cell proliferation. Instead, there was just enhancement of the ligand-induced response. Also, a major function of serine-118 phosphorylation in this instance may be an increase in the pool of stabilized ER α , an increase sufficient to enhance the response to E2.

Based on the effect of the PI3-kinase inhibitor, LY294002, the results seemed at first to indicate that E2 stimulated ER α serine-118 phosphorylation via this pathway. However, unbeknownst to us at the time of these original experiments, LY294002 has also been shown to be a competitive inhibitor of E2 [51]. The apparent effect of this molecule on both PI3-kinase and ERK activation by E2 may therefore, simply be competition between LY294002

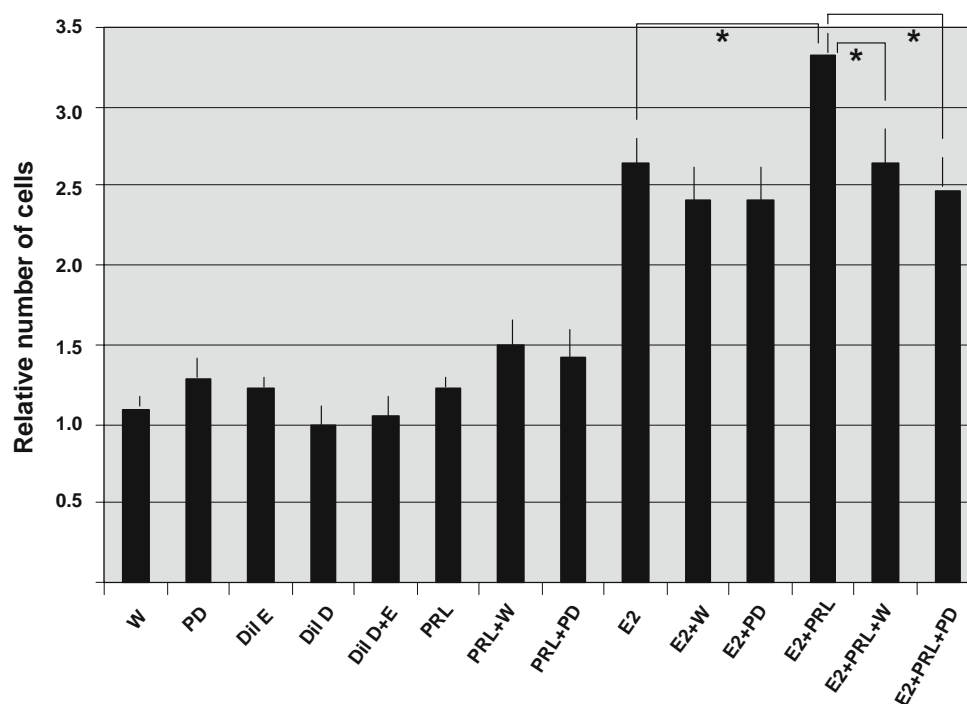


Fig. 8 Ability of kinase inhibitors to block the PRL enhancement of the effect of E2 on viable cell number. T47D cells were pre-incubated as before. The inhibitors or diluents (Dil) were added 2 h prior to the addition of hormones and the cells were then incubated for 3 days in phenol red-free medium containing 5% charcoal-stripped gelding serum. Preliminary experiments established the concentration of inhibitor that had no effect on basal proliferation. Dil E, ethanol; Dil

D, DMSO; Results for wortmannin are illustrated at 0.5 μ M and the results illustrated for PD are at 2 μ M. Relative cell number was determined by MTS assay. The results were normalized to the DMSO diluent control to combine experiments and are presented as the mean \pm SE. * $P < 0.05$. All abbreviations are as previously mentioned

and E2 for the receptor. This interpretation is supported by the fact that wortmannin, a second PI3-kinase inhibitor, had little to no effect on serine-118 phosphorylation, while clearly inhibiting phosphorylation of the downstream target of PI3-kinase, Akt. Interpretation of other authors' work implicating PI3-kinase in serine-118 phosphorylation by E2 may also be complicated by the use of LY294002. Although E2 stimulation does promote the formation of a ternary complex between ER α , Src and the catalytic subunit of PI3-kinase [10], the formation of this ternary complex has not been linked to serine-118 phosphorylation. Instead, the formation of this complex has been linked to longer-term effects of E2 on the cell cycle that may, as mentioned above, necessitate the phosphorylation of other sites on ER α .

Others have demonstrated rapid E2 activation of Akt and MAP kinase. Based on a time course produced by Castoria et al. [10], we may have missed the Akt activation since it peaks at 5–10 min. We did observe a slight activation of ERK at the 30 min time point, but the degree of activation was much more pronounced at 24 h. Nevertheless, indirect inhibition of Akt and ERK phosphorylation with wortmannin, or ERK phosphorylation with PD98059, had no effect on E2-stimulated serine-118 phosphorylation. While work with pharmacological inhibitors has its limitations, results obtained in the analysis of PRL signaling with the same inhibitors in the same time frame, would suggest that our interpretation is correct. Moreover, our results corroborate work published by Murphy et al. [52] who used the MAP kinase inhibitor, U0126, for similar studies. Thus, while E2 may activate PI3-kinase, Akt and ERK, our work suggests that these enzymes are not significant in the regulation of serine-118 phosphorylation. More recently, Cdk7, IKK α and GSK3 have been suggested as candidate kinases [22, 53, 54] and Murphy et al. have found evidence in support of IKK α , but not Cdk7 [52]. In contrast to E2, the effect of PRL on serine-118 phosphorylation appears to be mediated through the MAP kinase and PI3-kinase pathways. Both signals are independently initiated at the PRL receptor since the inhibitors had no crossover inhibitory effects. The route of activation of these kinases by PRL is, however, unclear at present. Results that are not presented demonstrated an unexpected degree of complexity revealed by the use of different concentrations of the Jak2 inhibitor, AG490, that will take some time to unravel. Nevertheless, whatever lies upstream of PI3-kinase and ERK in the PRL signaling pathway to serine-118 phosphorylation, the important point to be made here is that E2 and PRL clearly use different signaling pathways to achieve serine-118 phosphorylation.

In addition to different signaling, E2 and PRL employed different mechanisms to achieve down-regulation of ER α

in the 24 h time frame of most of our experiments; proteasomal degradation for E2 and an effect on mRNA levels for PRL. Based on the inhibitor studies, which showed very similar results for PRL at the mRNA and protein levels, it appears that signaling to effects at each potential level of regulation uses the same pathways. In addition, the degree of inhibitory effect of PRL and recovery with the inhibitors was very similar at the mRNA and protein levels, suggesting that regulation of mRNA levels accounted for all of the down-regulation. Continued analysis beyond 24 h showed E2 to also affect mRNA levels (data not shown), a finding previously reported by others [21].

Given that both PRL and E2 initiated serine-118 phosphorylation, which can be generally thought of as having a positive effect on ER-mediated functions, and both also down-regulated the total amount of ER α in the cells, the net outcome of PRL treatment on E2-related parameters such as cell proliferation was difficult to predict. Both PRL and E2 have been reported to increase the proliferation of a variety of human breast cancer cell lines, although the reported extent of the response to PRL has varied widely, even with the same cell line [4]. Many explanations for varied degrees of response to PRL are possible including the relative production of autocrine PRL by the cells, the number and types of PRL receptors expressed, the differential expression of signaling intermediates, and genetic instability causing variation in cell lines from lab to lab. In the current work, we present evidence that the co-existent steroidal environment can also be important for the ability of PRL to increase cell number. In our hands, PRL alone had no effect on T47D cell number, whereas others using E2-depleted culture conditions were able to show an effect [39, 50]. The reason for this is unclear at present, but could be related to the complete absence of serum in the experiments of others which lead to an anti-apoptotic effect of PRL. Regardless of some additional potential anti-apoptotic effect of PRL alone, the current experiments definitively show PRL enhancement of growth stimulation by E2. In addition, they show that inhibition of the same signaling pathways that resulted in serine-118 phosphorylation of ER α in response to PRL also inhibited the enhancement of E2-stimulated cell proliferation seen when PRL was added to the incubation. Thus, PRL can have an important effect on cell number in some breast cancers that is not obvious from the study of PRL alone. Moreover, activation of the PRL receptor can be added to the other growth factor receptor pathways that can influence E2 promotion of breast cancer.

In conclusion, the data support important crosstalk between PRL and E2 in the regulation of cell number in at least one widely-used ER positive breast cancer cell line.

Acknowledgments This work was supported by a grant from the California Breast Cancer Research Program, 10PB-0127. Y.C. was a recipient of a Cancer Federation award during this period. K.H. was supported by an individual fellowship from the DOD Breast Cancer Research Program, BC0501103.

References

- Arpino G, Wiechmann L, Osborne CK, Schiff R (2008) Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance. *Endocr Rev* 29:217–233
- Blank EW, Wong PY, Lakshmanaswamy R, Guzman R, Nandi S (2008) Both ovarian hormones estrogen and progesterone are necessary for hormonal mammary carcinogenesis in ovariectomized ACI rats. *Proc Natl Acad Sci USA* 105:3527–3532
- Chen GG, Zeng Q, Tse GMK (2008) Estrogen and its receptors in cancer. *Med Res Rev* 28:954–974
- Clevenger CV, Furth PA, Hankinson SE, Schuler LA (2003) The role of prolactin in mammary carcinoma. *Endocr Rev* 24:1–27
- Lakshmanaswamy R, Guzman RC, Nandi S (2008) Hormonal prevention of breast cancer: significance of promotional environment. *Adv Exp Med Biol* 617:469–475
- Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM (1995) The nuclear receptor superfamily: the second decade. *Cell* 83:835–839
- Tsai MJ, O'Malley BW (1994) Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* 63:451–486
- Jensen EV (1991) Overview of the nuclear receptor family. In: Parker M (ed) *Nuclear hormone receptors*. Academic Press, London, pp 1–13
- Shiau AK, Barstad D, Loria PM, Cheng L, Kishner PJ, Agard DA, Greene GL (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* 95:927–937
- Castoria G, Migliaccio A, Bilancio A, Di Domenico M, deFalco A, Lombardi M, Fiorentino R, Varrichio L, Barone MV, Auricchio F (2001) PI3-kinase in concert with Src promotes the S-phase entry of oestradiol-stimulated MCF-7 cells. *EMBO J* 20:6050–6059
- Kelly MJ, Levin ER (2001) Rapid actions of plasma membrane estrogen receptors. *Trends Endocrinol Metab* 12:152–156
- Song RX, McPherson RA, Adam L, Bao Y, Shupnik M, Kumar R, Santen RJ (2002) Linkage of rapid estrogen action to MAPK activation by ER α -Shc association and Shc pathway activation. *Mol Endocrinol* 16:116–127
- Castano E, Chen CW, Vorojeikina DP, Notides AC (1998) The role of phosphorylation in human estrogen receptor function. *J Steroid Biochem Mol Biol* 65:101–110
- Endoh H, Maruyama K, Masuhiro Y, Koayashi Y, Goto M, Tai H, Yanagisawa J, Metzger D, Hashimoto S, Kato S (1999) Purification and identification of p68 RNA helicase acting as a transcriptional coactivator specific for the activation function 1 of human estrogen receptor α . *Mol Cell Biol* 19:5363–5372
- Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D, Chambon P (1995) Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* 270:1491–1494
- Lavinsky RM, Jepsen K, Heinzel T, Torchia J, Mullen TM, Schiff R, Del-Rio AL, Ricote M, Ngo S, Gemsch J, Hilsenbeck SG, Osborne CK, Glass CK, Rosenfeld MG, Rose DW (1998) Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. *Proc Natl Acad Sci USA* 95:2920–2925
- Mendez P, Garcia-Segura LM (2006) Phosphatidylinositol 3-kinase and glycogen synthase kinase 3 regulate estrogen receptor-mediated transcription in neuronal cells. *Endocrinology* 147:3027–3039
- Migliaccio A, DiDomenico M, Castoria G, deFalco A, Bontempo P, Nola E, Auricchio F (1996) Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. *EMBO J* 15:1292–1300
- Migliaccio A, Piccolo D, Castoria G, Di Domenico M, Bilancio A, Lombardi M, Gong W, Beato M, Auricchio F (1998) Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. *EMBO J* 17:2008–2018
- Migliaccio A, Castoria G, DiDomenico M, deFalco A, Bilancio A, Lombardi M, Barone MV, Ametrano D, Zannini MS, Abbondanza C, Auricchio F (2000) Steroid-induced androgen receptor-oestradiol receptor beta-Src complex triggers prostate cancer cell proliferation. *EMBO J* 19:5406–5417
- Valley CC, Solodin NM, Powers GL, Ellison SJ, Alarid ET (2008) Temporal variation in estrogen receptor- α protein turnover in the presence of estrogen. *J Mol Endocrinol* 40:23–34
- Medunjanin S, Hermani A, De Servi B, Grisouard J, Rinke G, Mayer D (2005) Glycogen synthase kinase 3 interacts with and phosphorylates estrogen receptor α and is involved in the regulation of receptor activity. *J Biol Chem* 280:33006–33014
- Nirmala PB, Thampan RV (1995) Ubiquitination of the rat uterine estrogen receptor: dependence on estradiol. *Biochem Biophys Res Commun* 213:24–31
- Pakdel F, LeGoff P, Katzenellenbogen BS (1993) An assessment of the role of domain F and PEST sequences in estrogen receptor half-life and bioactivity. *J Steroid Biochem Mol Biol* 46:663–672
- Nawaz Z, Lonard DM, Dennis AP, Smith CL, O'Malley BW (1999) Proteasome-dependent degradation of the human estrogen receptor. *Proc Natl Acad Sci USA* 96:1858–1862
- Campbell GS, Argetsinger LS, Ihle JN, Kelly PA, Rillema JA, Carter-Su C (1994) Activation of JAK2 tyrosine kinase by prolactin receptors in Nb2 cells and mouse mammary gland explants. *Proc Natl Acad Sci USA* 91:5232–5236
- Rui H, Kirken RA, Farrar WL (1994) Activation of receptor-associated tyrosine kinase JAK2 by prolactin. *J Biol Chem* 269:5364–5368
- Tan D, Johnson DA, Wu W, Zeng L, Chen YH, Chen WY, Vonderhaar BK, Walker AM (2005) Unmodified prolactin (PRL) and S179D PRL-initiated bioluminescence resonance energy transfer between homo- and hetero-pairs of long and short human PRL receptors in living human cells. *Mol Endocrinol* 19:1291–1303
- Zheng J, Koblinski JE, Dutson LV, Feeney YB, Clevenger CV (2008) Prolyl isomerase cyclophilin A regulation of janus-activated kinase 2 and the progression of human breast cancer. *Cancer Res* 68:7769–7778
- Goupille O, Barnier JV, Guibert B, Paly J, Djiane J (2000) Effect of PRL on MAPK activation: negative regulatory role of the C-terminal part of the PRL receptor. *Mol Cell Endocrinol* 159:133–146
- Pezet A, Ferrag F, Kelly PA, Edery M (1997) Tyrosine docking sites of the rat prolactin receptor required for association and activation of Stat5. *J Biol Chem* 272:25043–25050
- Ali S, Ali S (1998) Prolactin receptor regulates Stat5 tyrosine phosphorylation and nuclear translocation by two separate pathways. *J Biol Chem* 273:7709–7716
- Clevenger CV, Ngo W, Sokol DL, Luger SM, Gewirtz AM (1995) Vav is necessary for prolactin-stimulated proliferation and is translocated into the nucleus of a T-cell line. *J Biol Chem* 270:13246–13253

34. Gouilleux F, Wakao H, Mundt M, Groner B (1994) Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription. *EMBO J* 13: 4361–4369
35. Clevenger CV, Torigoe T, Reed JC (1994) Prolactin induces rapid phosphorylation and activation of prolactin receptor-associated RAF-1 kinase in a T-cell line. *J Biol Chem* 269:5559–5565
36. Das R, Vonderhaar BK (1996) Involvement of SHC, GRB2, SOS and RAS in prolactin signal transduction in mammary epithelial cells. *Oncogene* 13:1139–1145
37. Erwin RA, Kirken RA, Malabarba MG, Farrar WL, Rui H (1995) Prolactin activates Ras via signaling proteins SHC, growth factor receptor bound 2, and son of sevenless. *Endocrinology* 136:3512–3518
38. Peters CA, Maizels ET, Robertson MC, Shiu RP, Soloff MS, Hunzicker-Dunn M (2000) Induction of relaxin messenger RNA expression in response to prolactin receptor activation requires protein kinase C delta signaling. *Mol Endocrinol* 14: 576–590
39. Acosta JJ, Munoz RM, Gonzalez L, Subtil-Rodriguez A, Dominguez-Caceres MA, Garcia-Martinez JM, Calcabrini A, Lazaro-Trueba I, Martin-Perez J (2003) Src mediates prolactin-dependent proliferation of T47D and MCF7 cells via the activation of focal adhesion kinase/Erk1/2 and phosphatidylinositol 3-kinase pathways. *Mol Endocrinol* 17:2268–2282
40. Berlanga JJ, Fresno Vara JA, Martin-Perez J, Garcia-Ruiz JP (1995) Prolactin receptor is associated with c-src kinase in rat liver. *Mol Endocrinol* 9:1461–1467
41. Clevenger CV, Medaglia MV (1994) The protein tyrosine kinase P59fyn is associated with prolactin (PRL) receptor and is activated by PRL stimulation of T-lymphocytes. *Mol Endocrinol* 8:674–681
42. Fresno Vara JA, Caceres MA, Silva A, Martin-Perez J (2001) Src family kinases are required for prolactin induction of cell proliferation. *Mol Biol Cell* 12:2171–2183
43. Mangoura D, Pelletiere C, Leung S, Sakellaridis N, Wang DX (2000) Prolactin concurrently activates src-PLD and JAK/Stat signaling pathways to induce proliferation while promoting differentiation in embryonic astrocytes. *Int J Dev Neurosci* 18:693–704
44. al-Sakkaf KA, Dobson PR, Brown BL (1997) Prolactin induced tyrosine phosphorylation of p59fyn may mediate phosphatidylinositol 3-kinase activation in Nb2 cells. *J Mol Endocrinol* 19:347–350
45. Yamauchi T, Kaburagi Y, Ueki K, Tsuji Y, Stark GR, Kerr IM, Tsushima T, Akanuma Y, Komuro I, Tobe K, Yazaki Y, Kadowaki T (1998) Growth hormone and prolactin stimulate tyrosine phosphorylation of insulin receptor substrate-1, -2, and -3, their association with p85 phosphatidylinositol 3-kinase (PI3-kinase), and concomitantly PI3-kinase activation via JAK2 kinase. *J Biol Chem* 273:15719–15726
46. Wang Y-F, Jia H, Walker AM, Cukierman S (1992) K-current mediation of prolactin-induced proliferation of malignant (Nb2) lymphocytes. *J Cell Physiol* 152:185–189
47. Chen TJ, Kuo C-YB, Tsai KF, Liu J-W, Chen D-Y, Walker AM (1998) Development of recombinant human prolactin receptor antagonists by molecular mimicry of the phosphorylated hormone. *Endocrinology* 139:609–616
48. Huang KT, Chen YH, Walker AM (2004) Inaccuracies in MTS assays: major distorting effects of medium, serum albumin, and fatty acids. *Biotechniques* 37:406–412
49. Weigel NL, Zhang Y (1998) Ligand-independent activation of steroid hormone receptors. *J Mol Med* 76:469–479
50. Philips N, McFadden K (2004) Inhibition of transforming growth factor-beta and matrix metalloproteinases by estrogen and prolactin in breast cancer cells. *Cancer Lett* 206:63–68
51. Pasapera Limon AM, Herrera-Munoz J, Gutierrez-Sagal R, Ulloa-Aguirre A (2003) The phosphatidylinositol 3-kinase inhibitor LY294002 binds the estrogen receptor and inhibits 17 β -estradiol-induced transcriptional activity of an estrogen sensitive reporter gene. *Mol Cell Endocrinol* 200:199–202
52. Murphy LC, Weitsman GE, Skliris GP, The EM, Li L, Peng B, Davie JR, Ung K, Niu Y-L, Troup S, Tomes L, Watson PH (2006) Potential role of estrogen receptor α (ER α) phosphorylated at serine 118 in human breast cancer in vivo. *J Steroid Biochem Mol Biol* 102:139–146
53. Chen D, Washbrook E, Sarwar N, Bates GJ, Pace PE, Thirunavakkarasu V, Taylor J, Epstein RJ, Fuller-Pace FV, Egly JM, Coombes RC, Ali S (2002) Phosphorylation of human estrogen receptor alpha at serine 118 by two distinct signal transduction pathways revealed by phosphorylation-specific antisera. *Oncogene* 21:4921–4931
54. Park K, Krishnan V, O'Malley B, Yamamoto Y, Gaynor R (2005) Formation of an IKK α -dependent transcription complex is required for estrogen receptor-mediated gene activation. *Mol Cell* 18:71–82

Long-term increased expression of the short form 1b prolactin receptor in PC-3 prostate cancer cells decreases cell growth and migration, and causes multiple changes in gene expression consistent with reduced invasive capacity.

Huang KT and Walker AM. The Prostate, under revision.

Background: Inhibition of prostate cancer cell growth with a prolactin (PRL) receptor-specific antagonist, S179D PRL, up-regulates the short receptor isoform, SF1b. We also identified a naturally occurring form of the SF1b receptor (as Δ S2 SF1b), lacking half of the extracellular domain, to be constitutively active.

Method: In the current study, we have taken the advantage of the constitutive activity and established PC-3 prostate cancer cell lines with increased expression of Δ S2 SF1b.

Results: S179D PRL dose-dependently increases both the intact and Δ S2 forms of SF1b in PC-3 cells. Stable expression of Δ S2 SF1b in PC-3 resulted in decreased cell growth compared to the vector control cells. Although with no apparent changes in expression levels, Δ S2 SF1b expressing cells showed different distribution of E-cadherin. Increased expression of Δ S2 SF1b also decreased cell migration, as assessed in wound healing and TranswellTM assays. Quantitative RT-PCR analyses on cell migration-related genes showed significant down-regulation of proteases MMP-9 and uPA, and up-regulation of protease inhibitors PAI-1 and PAI-2. Also down-regulated were growth factors bFGF, VEGF, and the adherens junction component β -catenin. We also demonstrated that the observed effects of increased Δ S2 SF1b were not a result of a dominant negative effect between autocrine PRL and the endogenous long form receptor.

Conclusion: We conclude that increased expression of the hormone-inducible Δ S2 SF1b PRL receptor decreases cell growth, migration, and invasive capacity and can be potentially beneficial.

A naturally occurring, inducible, constitutively active isoform of the human prolactin receptor, Δ S2 SF1b, reduces proliferation and migration in human prostate cancer cells

Huang KT and Walker AM. (2006) The American Society for Cell Biology 46th Annual Meeting.

We previously demonstrated that a molecular mimic of phosphorylated prolactin (PRL), S179D PRL, inhibited prostate tumor growth. In part this is through antagonism of a PRL autocrine growth loop, and in part this is through up-regulation of one of the short prolactin receptor (PRLR) isoforms, SF1b, resulting in an increase in p21 and vitamin D receptor expression. In the current study, we identified a novel, naturally occurring form of PRLR, which lacks about half of the extracellular domain and is active in the absence of ligand. Stable prostate cancer cell lines expressing this constitutively active receptor, designated Δ S2 SF1b, were established. These cells grew more slowly than their control transfected counterparts. Overexpression of Δ S2 SF1b also inhibited cell migration as analyzed by wound healing and transwell assays. Semi-quantitative and real-time RT-PCR analysis revealed that Δ S2 SF1b expression up-regulated the protease inhibitors TIMPs 1&2 and PAIs 1&2, and down-regulated both the cognate proteases MMPs and uPA, and the growth factors bFGF and VEGF. Immunofluorescent staining of a cell junction component, E-cadherin, showed more cell-cell contacts in Δ S2 SF1b cells. Since S179D PRL upregulates intact SF1b and this is associated with decreased migration of endothelial cells, we investigated the effect of S179D PRL on prostate cancer cell migration and invasion. Treatment of LNCaP cells with S179D PRL inhibited cell migration in the transwell assays. Analysis of gene expression showed that S179D PRL up-regulated both the intact and Δ S2 SF1b, extending the previous study to include the Δ S2 version. Also upregulated were the TIMPs and PAIs. We conclude that overexpression of the constitutively active short PRLR isoform, Δ S2 SF1b, inhibits cell migration and likely reduces invasive properties. Similar results can be achieved by treatment with S179D PRL, which up-regulates the expression of this particular isoform.

Evidence suggesting that prolactin functions in the mammary duct.

Ueda E, Huang KT, Nguyen V and Walker AM. (2008) The 2008 Gordon Research Conference: Prolactin and Growth Hormone Family.

Development and function of the mammary gland involves the orchestrated activities of a number of steroid and peptide hormones. Since a large part of the activity of steroid hormones is via cytoplasmic/nuclear receptors, the subcellular distribution of steroid receptors would not be expected to be very telling in regard to the source of activating ligand. Signals from prolactin (PRL), on the other hand, are mediated through plasma membrane receptors and hence one would expect the distribution of receptors into apical or basolateral regions of the membrane to reflect the source of stimulus.

In the present study, we examined localization of PRL receptors (PRLRs) in the virgin, lactating and involuting mouse mammary gland. Antibodies recognized either the extracellular domain or intracellular regions specific for the long or one short form, PR3. The PR3 short receptor has similarities in sequence to the short rat receptor. Since the rat has only one short receptor, it seemed likely that PR3 was the most relevant to common mammary gland functions. In virgin glands, there was heavy apical localization of both receptor types on the epithelial cells and this primarily apical localization continued through day 2 of lactation. This result is exactly the opposite of what one would expect if the cells were designed to respond primarily to PRL in the bloodstream. Instead, the localization suggests a dependence on autocrine PRL, although because junctions between epithelial cells are leaky at these times, circulating PRL could contribute or even substitute for autocrine PRL. Whether PRL is autocrine or endocrine in origin, the apical localization of the receptors demonstrates that it interacts with its receptors in the specialized and somewhat restricted micro-environment of the duct lumen. By day 7 of lactation when the junctions are tight, the receptors were evenly distributed on each face, a situation which continued through day 12 and which suggests a dependence on pituitary PRL for full lactational function. At day 18, the receptors were again primarily apical.

Also examined was expression of PRLRs during polarization and junction formation in HC11 cells. Long receptors were present on cells regardless of junction formation, whereas PR3 receptors only appeared co-incidentally with junction proteins. Transfection of HC11 cells with human receptors showed the SF1b, but not SF1a or the long form to accelerate junction formation, as assessed by the development of transepithelial resistance. Once junctions were present, receptors were confined to the apical surface and were shown to be functional by analysis of Stat5 and ERK phosphorylation. Essentially no signal was generated from the basolateral surface. These results suggest functioning of PRL primarily within the duct lumen (except in mid-lactation), a micro-environment with very different properties to the basolateral stroma.

Different prolactin receptors mediate different functions in breast cancer cells suggesting the importance of a short and a soluble form to normal breast health.

Huang KT, Tan D and Walker AM. (2008) Department of Defense Breast Cancer Research Program – Era of Hope 2008.

Prolactin (PRL) is a hormone that has been implicated as a contributing factor in the incidence and progression of breast cancer. Several different isoforms of the PRL receptor (PRLR) have been discovered, most of which are produced by alternative splicing of a single gene product. The major isoforms include a long form (LF), an intermediate form, and two short forms (SF1a and SF1b), each of which have the same extracellular sequence (comprised of S1 and S2 regions), but different intracellular signaling domains. We have discovered naturally-occurring forms of each of these PRLRs, which maintain the same intracellular signaling regions, but lack about half of the extracellular domain. Importantly, these receptors proved to be constitutively active. The objective of our study was to use these constitutively active receptors (designated Δ S2) to determine the individual roles of the different PRLRs in mammary cells. In transiently transfected human breast cancer cells (T-47D), expression of Δ S2LF increased cell number, whereas both Δ S2 LF and Δ S2 SF1a increased endogenous β -casein gene expression. In addition, our data show the other short isoform, Δ S2 SF1b, inhibited cell proliferation and migration in a copy number-related fashion when stably overexpressed in several cell types. In an effort to further evaluate the effect of this apparently beneficial isoform, stable T-47D breast cancer cells expressing Δ S2 SF1b under the control of a tetracycline-responsive promoter were produced. Analysis showed that administration of tetracycline and the subsequent overexpression of Δ S2 SF1b induced prolonged ERK activation in the absence of ligand. Overexpression of Δ S2 SF1b also upregulated the cell cycle inhibitor, p21, and the milk protein, β -casein, suggesting both an anti-proliferative and pro-differentiative role for this receptor, and by inference the regular SF1b receptor in the presence of ligand. In the course of our studies, we also identified another truncated PRLR isoform. Sequence analysis revealed an exon 6 deleted transcript. This deletion creates a frameshift in the open reading frame resulting in a foreshortened soluble receptor essentially composed of just half of the extracellular domain. This form, designated SS1 (for soluble S1), has been previously described at the mRNA level by Laud et al. (Int J Cancer. 2000; 85:771-6). RT-PCR compared expression of transmembrane receptors to SS1 in tumor samples from patients with invasive ductal carcinoma versus histologically normal contiguous regions from the same patients. Preliminary results from four pairs showed a larger complement of SS1 in the normal regions, suggesting a beneficial role of this isoform. SS1 protein was detected in T-47D culture medium by immunoprecipitation. Co-immunoprecipitation demonstrated binding of PRL and SS1. Furthermore, SS1 conditioned medium, produced by the overexpression of SS1 in HEK293 cells, modulated PRL-induced signaling, prolonging ERK activation and reducing Stat5 activation. Thus far, the data suggest that SS1 is a soluble PRL binding protein that has the capacity to modulate PRL signaling. There is a correlation between loss of SS1 and the development of invasive ductal carcinoma, a result which suggests beneficial aspects to increased expression of SS1. Our data with Δ S2 SF1b supports the conclusion that increased expression of this, or the regular SF1b with ligand, would also be beneficial in breast cancer patients.

S179D prolactin blocks estradiol-induced breast cancer cell proliferation in a manner duplicated by signaling from the short (SF1b) prolactin receptor.

Huang KT, Chen YH, Tan D and Walker AM. (2009) The Endocrine Society 91st Annual Meeting.

Elevated levels of (and/or increased responses to) both prolactin (PRL) and estradiol (E2) have each been implicated as contributing factors in the development of breast cancer, but relatively little is known about how these two hormones interact at the level of mammary epithelium. We have previously demonstrated that PRL enhances the proliferative effect of E2 on T-47D human breast cancer cells, and causes sustained phosphorylation of estrogen receptor (ER) α at serine-118, the phosphorylation site most important for transcriptional activity and stabilization of this receptor. In the current study, our focus is a molecular mimic of phosphorylated human PRL, S179D PRL. Treatment with S179D PRL (100 ng/ml) completely blocked E2-induced (1nM) cell proliferation in T-47D cells, reduced E2-induced serine-118 phosphorylation of ER α , and down-regulated E2-responsive genes (e.g. cyclin D1). An earlier similar experiment with MCF7 cells also showed the ability of S179D PRL to block E2 stimulation of this cell line. We have previously described the pro-differentiative properties of the short PRL receptor isoform, SF1b, and its up-regulation with S179D PRL. In contrast to S179D PRL, E2 treatment halved SF1b expression in T-47D cells, as measured by real-time RT-PCR. Decreased expression of SF1b was also observed in tumor samples from patients with invasive ductal carcinoma versus histologically normal contiguous regions. T-47D cells express both long and short PRL receptors and S179D PRL interacts with each. To determine whether the effect of S179D PRL on E2-mediated effects could be duplicated by signaling from the SF1b receptor, we developed T-47D cells with inducible expression of a constitutively active form of SF1b (Δ S2 SF1b). This constitutively active SF1b does not interfere with PRL-initiated Stat5 activation, as assessed using a Stat5-responsive luciferase construct, and does not heterodimerize with the long receptor, as assessed by BRET analysis. Induced expression of Δ S2 SF1b decreased E2-stimulated cell growth, phosphorylation of Akt and its downstream kinase, GSK3 β . These results suggest beneficial effects of the SF1b receptor in terms of limiting breast cancer cell proliferation beyond those brought about by inhibition of Stat5 activation at the long receptor. These beneficial effects can be recapitulated by treatment with S179D PRL.